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A Molecular Epidemiological Investigation of Group B Streptococcus

A thesis submitted to the Open University for the degree of
Doctor of Philosophy

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Abstract.

A multilocus sequence typing (MLST) system for group B streptococcus (GBS) has been developed and validated on a global collection of human GBS strains isolated from carriage and invasive disease.

A carriage study was performed, over a 3-year period, to establish the rate of carriage of GBS in pregnant women in Oxford, UK. Invasive isolates were collected, prospectively and retrospectively over a similar time period.

Twenty-one percent of women studied were asymptomatic carriers of GBS. The incidence of invasive GBS was 0.9/1000 live births in neonates and 6.1/100,000 population >60 years.

The population structure of GBS is best depicted, using MLST, as a network of related clusters indicating the presence of recombinational events occurring in the population that interfere with a tree like branching structure of the population.

A single hypervirulent clone of GBS (ST-17 complex) is responsible for an excess of neonatal disease in Oxford (odds ratio 3.4). The possibility that a factor other than capsular type III may be responsible for virulence of this clonal complex in neonates is raised. Intriguingly this clonal complex was unique among human lineages in that it has emerged from bovine GBS. It was not however associated with increased invasiveness amongst adult (> 60 years).

Further study of this hypervirulent clone of GBS is likely to contribute to the understanding of the pathogenesis of neonatal GBS disease.

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Midwives at the John Radcliffe and Horton Hospitals and Jan Burry (anaesthetic nurse) approached pregnant women and carried out recruitment, collection of swabs and filled in questionnaires (Chapter 4).

Microbiology Methods:

Karen Oliver (Research Assistant) assisted in the microbiological work required for isolation of group B streptococci for the carriage study (Chapter 4) and invasive GBS study (Chapter 3). In addition Karen maintained freezer files of strains collected.

Molecular methods:

Karen Oliver helped to carry out molecular work including DNA extraction and MLST methods for collections of GBS for the carriage study and invasive neonatal study (Chapter 7).

Naiel Bisharat carried out molecular work including DNA extraction and MLST methods for collections of GBS for the bovine study (Chapter 9).

Jo Barry helped to carry out molecular work including DNA extraction and MLST methods for collections of GBS for the adult over 60 years study (Chapter 8).

Computational methods:

Man-Suen Chan developed and maintained the databases for STARS and the GBS website. Carol Bridgeford assisted in the development of the Access databases.

List of Abbreviations.

<i>adhP</i>	alcohol dehydrogenase
<i>atr</i>	amino acid transporter
BAA	Bile-aesculin agar
BURST	Based-upon Related Sequence Types
CDC	Centers for Disease Control and Prevention, Atlanta, USA
CI	95% Confidence intervals around the mean
CS	Caesarean section
CSF	Cerebro-spinal fluid
<i>cysK</i>	cysteine synthetase A
D	Index of diversity
DNA	Deoxyribosenucleic acid
dNTP	Deoxyribose nucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ET	Electrophoretic type
GBS	Group B Streptococcus
<i>glcK</i>	glucose kinase
<i>glnA</i>	glutamine synthetase
IAP	Intrapartum antibiotic prophylaxis
IE	Infective endocarditis
IV	Intravenous route of administration
JRH	John Radcliffe Hospital
MLEE	Multilocus enzyme electrophoresis
MLST	Multilocus sequence typing
NaCl	Sodium Chloride
ND	Not done
NT	Not typable
NZ	New Zealand
OR	Odds ratio
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PFGE	Pulse field gel electrophoresis
<i>pgm</i>	phosphoglucomutase
<i>pheS</i>	phenylalanyl tRNA synthetase
PO	Per oral route of administration
PROM	Prolonged rupture of amniotic membranes
<i>pscb</i>	ribose-phosphate pyrophosphokinase
<i>purK</i>	phosphoribosylglycinamide formyltransferase
RAPD	Random amplified polymorphic DNA
RDP	Restriction digest pattern
<i>ribF</i>	riboflavin biosynthesis protein
S	Segregating site
<i>sdhA</i>	serine dehydratase
SEM	Standard error of the mean
ST	Sequence type
TBE	Tris borate EDTA
<i>tkt</i>	transketolase
UPGMA	Unweighted pair group method with arithmetic means
UTI	Urinary tract infection

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Chapter 1: Introduction

1.1 GROUP B STREPTOCOCCUS:

1.1.i *Historical perspective:*

The first descriptions of human disease caused by Lancefield group B β -haemolytic streptococcus, *Streptococcus agalactiae*, were fatal cases of puerperal sepsis in 1935¹. Occasional reports appeared in the literature over the next few decades, but it was only in the 1970s, when a dramatic increase in cases was reported, that group B streptococcus (GBS) was recognised as the leading cause of human neonatal sepsis in geographically diverse regions²⁻⁴.

1.1.ii *Microbiology:*

Streptococcus agalactiae is the species designation of GBS. It is a facultative anaerobe and a Gram-positive coccus occurring in pairs or short chains. Colonies on agar supplemented with horse or sheep blood appear as mucoid, are coloured grey, buff or white, are 3-4mm across and are surrounded by a thin rim of β -haemolysis.

Definitive identification requires detection of the group B carbohydrate antigen usually by serological methods, first described by Rebecca Lancefield⁵.

Commercial kits based on latex agglutination are commonly used in clinical microbiology laboratories.

Other biochemical tests have been employed for presumptive identification of GBS including: resistance to bacitracin, hydrolysis of sodium hippurate broth, failure of hydrolysis of bile-aesculin agar (BAA), pigment production on certain media in anaerobic growth conditions^{6,7} and production of CAMP factor⁸.

1.1.iii Typing methods for GBS:

There is a type-specific polysaccharide capsule on the GBS surface⁹. Nine capsular types have been characterised, IA, IB, II to VIII and have traditionally been the method of distinguishing between strains.

Several molecular techniques including restriction digest pattern (RDP) typing¹⁰, pulse field gel electrophoresis (PFGE)¹¹ and multilocus enzyme electrophoresis (MLEE)^{12,13} have been used on collections of GBS, but there has been no universally accepted typing method other than capsular serotyping.

1.1.iv Virulence of GBS:

The role of type specific polysaccharides in virulence of GBS has been evaluated in murine¹⁴ and chick models¹⁵, where the amount of capsule was found to correlate with invasiveness. In addition, a capsule-free mutant strain of GBS was effectively cleared in a murine model¹⁶. The component monosaccharides of the GBS capsule are repeating units of galactose, glucose, N-acetylglucosamine and sialic acid¹⁷⁻²⁴. Sialic acid as a terminal side chain residue is a consistent feature and in the cases of type IA, IB and III polysaccharides there is a high degree of structural homology with two human serum glycoproteins, the M and N blood group substances²⁵. The type III capsule is structurally homologous and

immunochemically identical to the pneumococcal type 14 polysaccharide ²⁶.

Molecular mimicry of host surface structures resulting in poor recognition and opsonophagocytosis of the encapsulated bacteria is believed to account at least in part, for the virulence of GBS.

The haemolysins of GBS are an extracellular product of almost all strains and are active against the erythrocytes of several mammalian species ²⁷. Mutant GBS lacking haemolysin, however, did not differ in virulence to wild type in a neonatal rat model ²⁸. More recently, using a murine model, β -haemolysin induced nitric oxide production and septic shock.

Most strains of GBS elaborate C5a peptidase, a serine protease that inactivates the human complement component C5a ²⁹. It is thought that C5a peptidase contributes to virulence by preventing neutrophil accumulation at the site of infection ³⁰ and by promoting fibronectin binding. There is similarity between GBS C5a peptidase and a similar molecule in group A streptococcus ³¹.

Neuraminidase, possibly a hyaluronate lyase, active against host sialic acid substrates, may also be associated with virulence ¹³. GBS synthesise lipoteichoic acid and other teichoic acids that facilitate attachment to human cells ³².

1.1.v Epidemiology of human GBS infections:

1.1.v.a Asymptomatic colonisation. The primary reservoir for GBS is likely to be the lower gastrointestinal tract of men and women. Studies of GBS

carriage are difficult to compare directly because of differences in methodology, particularly the body sites sampled and the microbiological methods used. It is estimated that up to 30% of healthy adults are carrying the organism³³⁻³⁸.

Efficiency of identification of female genital GBS carriage increases when sampling is from the vulva or low vagina. Sampling of both lower genital and rectal (vagino-rectal) areas identifies 10-15% more carriers than if a single site is cultured³⁹⁻⁴¹. Isolation rates are significantly higher when broth rather than solid agar is used. Selective media⁴²⁻⁴⁴, containing antibiotics inhibitory to the normal flora, typically gentamicin 4-8 mcg/ml and nalidixic acid 15 mcg/ml³³ also increases the yield of GBS in culture.

Cultures performed 1 to 5 weeks before delivery are accurate in predicting GBS colonisation status at delivery in women at term. One study reported a positive predictive value of 87% and a negative predictive value of 96% when cultures were carried out in this time interval⁴⁵. Longitudinal studies of genital tract GBS colonisation in pregnant women (n=382) showed that carriage was chronic in 36%, transient in 20%, intermittent in 15% and indeterminate in 29%⁴⁶.

Several factors have been associated with an increased risk of GBS carriage in studies of pregnant and non- pregnant women. These factors include, diabetes mellitus⁴⁷, tampon use⁴⁸, age <20 years⁴⁹, Hispanic and African-American race⁵⁰ and intrauterine device use⁵¹. Factors that do not seem to affect prevalence of GBS carriage are use of oral contraceptives⁴⁰, marital status, sexually transmitted infections and number of sexual partners⁵².

1.1.v.b Transmission to the neonate. Exposure of the neonate to GBS in the maternal genital tract at delivery occurs by ascending infection through ruptured membranes *in utero*, or by contamination during passage through the birth canal. The mean rate of transmission of GBS from a carrier mother to her neonate is 50%, whereas only about 5% of neonates of culture-negative mothers become GBS colonised within 48 hours of life ^{53,54}. Vertical transmission appears to be more likely where there is heavy colonisation of the mother ⁵⁵. The neonatal colonisation tends to be of surface and mucous membrane sites and can persist for weeks ³⁹. Horizontal transmission of GBS to the neonate from hospital or community sources also occurs ⁵⁶.

Infants asymptotically colonised from a maternal source in the peripartum period have persistent carriage of GBS for weeks ^{57,58}. Although horizontal transmission of GBS to the neonate can occur in maternity units or in the community it appears to be unusual. In a study of 46 neonates GBS culture-negative on discharge from hospital, only 2 had mucous membrane carriage at 2 months of age ⁵⁷⁻⁵⁹.

1.1.v.c Neonatal Invasive disease. There are two distinct syndromes of neonatal GBS infection, termed early-onset (<7 days of age) and late-onset (7-90 days of age) sepsis. Prevalence of GBS bacteraemia is 0.5 ⁶⁰ to 3.7 ⁶¹ /1000 live births for early-onset and 0.3 ⁶⁰ to 1.8/1000 ⁴¹ live births for late-onset sepsis. In the USA, the incidence of early-onset disease has decreased by 65% from 1993 to 1998 from 1.7 to 0.6/1000 live births as a result of the use of

intrapartum antibiotic prophylaxis (IAP) ⁶². Stratification of attack rates of early-onset GBS infection by birth weight shows that lower birth weight categories have higher rates of infection ⁶³.

When probable, as well as definite neonatal infection (Table 2.5) is studied, the incidence rates of neonatal GBS infection are higher^{64.65}. Higher rates of early onset neonatal GBS disease, 1.5/1000⁶⁶, were described in the USA prior to the introduction of preventative strategies in 1994. This has now dropped to 0.5/1000⁶⁷ since those strategies have been introduced.

Table 1.1: Studies estimating early and late onset neonatal sepsis from 1990 to 2003.

Study	Year	Early-onset neonatal infection	Late-onset neonatal infection	Overall	Ref
Bedfordshire, UK	2000	1.15 (0.6- 1.7)	ND	ND	68
London, UK	2003	3.6* (1.9- 5.3)	ND	ND	65
Oxford, UK	1998	0.9* (0.7- 1.3)	ND	ND	69
London, UK	2001	2.6*	ND	ND	64
National, UK	2001	0.5 (0.5- 0.6)	0.3	0.8 (0.7-0.9)	60
Sunderland, UK	1999	0.95	0.47	1.42 (0.8-2.0)	70
Finland	2003	0.65 (0.6- 0.7)	ND	ND	71
USA	1990	1.5	0.3	1.8	72
USA	2000	0.5 (0.4-0.6)	ND	ND	67

Footnote to Table 1.1: Figures are expressed as number of cases/1000 live births of invasive neonatal GBS infection. Ninety-five percent confidence intervals are indicated in parentheses, where available. Infection was diagnosed by culture of GBS from a normally sterile site (definite infection).

*definite and probable GBS infection included in estimate of incidence.

Abbreviations: ND, not done.

Early onset GBS infection commonly presents as bacteraemia without a focus (25 to 40%), pneumonia (35 to 55%) and meningitis (5 to 10%). Hypotension and respiratory abnormalities are early signs. Other signs are lethargy, poor feeding, hypothermia, fever, abdominal distension, tachycardia and jaundice

^{73,74}. Infection occurring *in utero* may be associated with foetal asphyxia.

Meningitis can present in the same way as bacteraemia ⁷⁵, thus cerebrospinal fluid (CSF) examination is the only reliable means to exclude the diagnosis. Seizures may complicate meningitis. Radiological findings of pneumonia are indistinguishable from hyaline membrane disease ⁷⁶. Mortality rates are between 5-10% ^{73,74}. Features associated with a poor outcome are low APGAR score at birth, shock, coma, neutropaenia, pleural effusions, apnoea, delayed diagnosis and prematurity ^{77,78}.

Late onset GBS infection occurs in infants who typically have an unremarkable early neonatal history. Meningitis is the presentation of 35-40% of cases, with bacteraemia with no focus occurring with similar or higher frequency ⁴¹. Other, less common, manifestations of late onset infection are osteomyelitis, septic arthritis, cellulitis and adenitis ⁷⁹⁻⁸¹. Meningitis typically presents with fever, irritability, lethargy, poor feeding and tachypnoea. Complications include, seizures, status epilepticus and coma, and are markers of a poor outcome, with approximately 26% of cases being fatal ^{54,77}. Subdural empyema and hydrocephalus requiring shunting can also occur ⁸². Permanent moderate or severe neurological sequelae are present in about 21% of cases ⁸³.

Approximately 0.5 to 3% of neonatal patients have relapsed or recurrent GBS infection, occurring up to 4 months after treatment ⁸⁴⁻⁸⁶. Persistence of the organism on mucous membranes ⁸⁷ or in a deep site ^{88,89}, inadequate therapy and tolerance of GBS to penicillin ⁹⁰ are factors implicated in recurrence.

1.1.v.d GBS infection acquired during adulthood. More than two-thirds of GBS disease in the USA occurs in adults⁹¹. GBS infections commonly affect those with underlying medical conditions, especially diabetes mellitus. Other medical conditions, which predispose to GBS invasive infection are liver cirrhosis, malignancy, vascular disease and decubitus ulcers⁹². In studies, 70-93% of adult patients with GBS infection had one or more underlying medical illness^{62,93,94}.

The mean age of non-pregnant adults with invasive GBS disease is 60 years and elderly adults in nursing home accommodation appear to be at particular risk⁹⁵. Annual incidence rates of GBS disease in adults have been estimated in the USA as 4.1 to 7.2/100,000 population⁹⁵.

GBS is a common maternal pathogen in the perinatal period. It is responsible for chorioamnionitis, urinary tract infection, postpartum endometritis and wound infection after caesarean section (CS). It has been estimated that the attack rate for maternal GBS puerperal sepsis is 2/1000⁹⁶. Pregnant women account for 10-15% of cases of adult GBS bacteraemia⁷².

1.1.vi Bovine group B streptococcal infections:

GBS is a highly contagious pathogen of the bovine mammary gland. The organism can persist for many months in an individual and can spread to infect herds of dairy animals. It is unclear whether host-specific ecovars exist in cattle and humans. Contradictory reports indicate the absence or presence of a relationship between human and bovine GBS isolates.

Bovine mastitis has been induced artificially using both human and bovine-derived GBS strains⁹⁷. A study of Slovenian dairy farms, suggested that farm workers were carrying the same capsular serotypes of GBS as their cattle⁹⁸. Using MLEE, Musser and colleagues demonstrated that human and bovine GBS strains fell into the same lineages, although the numbers studied were small¹³. In contrast, RAPD (random amplified polymorphic DNA) typing of GBS revealed that there was little overlap between human carried and bovine mastitis causing GBS strains isolated in Canada⁹⁹. The exact relationship between human and bovine derived GBS is yet to be determined.

1.1.vii Treatment of human GBS disease:

GBS remains susceptible to penicillin and resistance has not been reported¹⁰⁰. Penicillin G is the drug of choice for the treatment of invasive infections. Other β -lactam antibiotics, including the carbapenems and many of the cephalosporins are active against GBS. Duration of therapy has not been extensively studied, but the traditional course of penicillin for neonatal bacteraemia is 10 days and longer courses (2-6 weeks) have been suggested where there is endocarditis, meningitis or osteoarticular disease. Initial therapy including synergistic aminoglycosides may be superior to β -lactams alone¹⁰¹. Interestingly, *in vitro* studies have suggested that chloramphenicol and rifampicin may antagonise the effect of β -lactam agents on GBS and are generally not recommended^{102,103}.

Increasing resistance to certain groups of antibiotics has occurred in GBS. In particular rates of tetracycline resistance have increased from 30% in 1950 to

90% in current times. Resistance to erythromycin and clindamycin have also increased and exceed 40% in Taiwan¹⁰⁴. A recent estimate of the prevalence of erythromycin resistance in UK GBS isolates is 10%.

1.1.viii Prevention of neonatal GBS disease:

The basic strategies in the prevention of neonatal GBS are directed either at eliminating exposure (chemoprophylaxis) or enhancing immunity (immunoprophylaxis).

1.1.viii.a Chemoprophylaxis. Early studies in the 1970s of administration of oral penicillin to GBS colonised women late in pregnancy proved to be ineffective in the prevention of invasive neonatal infection¹⁰⁵. The inherent difficulty of eradicating an organism with a reservoir in bowel flora is a likely explanation for this finding. Intravenous antibiotics administered during labour were investigated in 1979 and the interruption of transmission of GBS to the neonate was impressive¹⁰⁶. Further studies confirmed the findings and extended the beneficial effect of the intravenous antibiotics to the treated mothers^{55,107}, who suffered less GBS related infectious morbidity⁵⁵.

It is generally accepted that IAP with intravenous penicillin is the most effective method, in the current setting, of preventing neonatal early-onset GBS infection. The incidence of late-onset neonatal infection has not however been affected by this strategy. Over the past two decades, debate has continued as to which groups of women should be offered IAP. The two main strategies proposed in the CDC (Centres for Disease Control and Prevention, Atlanta, USA) consensus

guidelines³³ were based upon the results of vagino-rectal culture or assessment of risk-factors in the pregnant woman.

The culture-based approach identifies women for IAP following identification of GBS in vagino-rectal swabs cultured in selective broth taken at 35-37 weeks of pregnancy. The risk-factor approach identifies women to be offered IAP according to the presence of factors, which are known to increase the likelihood of neonatal GBS disease. The factors include onset of labour prior to 37 weeks of pregnancy, intrapartum fever and rupture of membranes more than 18 hours before delivery¹⁰⁸. In either strategy, a previous infected neonate and the finding of GBS bacteriuria are further factors identifying women for IAP. Following either strategy, approximately 25% of pregnant women are given IAP. The proportion of disease theoretically prevented by the culture-based approach is higher (85-90%) than that prevented by the risk factor approach (50-65%). Following implementation of these guidelines in the USA neonatal early onset GBS infection was reduced by 65% to 0.6/1000⁶².

1.1.viii.b Immunoprophylaxis. Vaccination of all women of child-bearing age either before or during pregnancy is the most promising strategy for the prevention of neonatal GBS infection^{109,110}. Human sera containing type III specific capsular antibody is protective against lethal challenge in animal models^{111,112}. Type-specific antibody is thought to be protective in human disease^{113,114}. Protection of the newborn could theoretically be achieved through immunisation of the mother. Purified capsular polysaccharides (IA, II, III, V) have been utilised in clinical trials in adults and

have been associated with an immune response^{115,116}. Protein conjugate capsular polysaccharide vaccines may have increased immunogenicity. Initial results of conjugate tetanus- toxoid polysaccharide vaccines have revealed them to be safe and immunogenic in adults^{117,118}. Other vaccines in early trials utilise ubiquitous GBS surface proteins, such as C5a peptidase¹¹⁹ and Sip protein¹²⁰.

1.2 POPULATION BIOLOGY OF PATHOGENIC BACTERIA:

1.2.i The clonal model of population structure of bacteria:

Pathogenic bacteria exist as populations, the members of which exhibit varying degrees of virulence. The study of evolution, epidemiology and population genetics can provide important insights into the origins and spread of pathogenic bacteria and is helpful in the design of effective public health interventions. In recent times, with the advent of automated high-throughput DNA (Deoxyribosenucleic acid) sequencing, closer study of the complexities of the evolution of bacterial populations is now possible¹²¹.

The clonal population structure is a popular paradigm for the exploration of bacterial population diversity. The basis of the model is that haploid bacteria reproduce asexually by binary fission and chromosomal variations occur by *de novo* mutations, leading to linkage disequilibrium between alleles at different loci. This contrasts with populations of sexual organisms where mutations are constantly re-assorted, resulting in linkage equilibrium, meaning that mutations at different sites occur in random combinations. Diversity can arise in asexual

populations by other mechanisms, such as the gain and loss of plasmids or the movement of insertion sequences. Differences in the frequencies of particular lineages in the population will occur over time as a result of stochastic events and selection. Bacterial populations are also subject to rapid expansions and bottlenecks, which can also reduce the diversity of clonal populations. While limited diversity is predicted under the clonal model, it is not the only reason why a bacterial population may be uniform and lack of diversity alone should not be used as proof of clonality¹²².

In reality, bacteria rarely conform entirely to the idealised clonal model¹²³. The complications of bacterial population biology arise from the fact that while bacteria are largely asexual, they possess a number of mechanisms for the horizontal transmission of genetic material and recombination, including plasmids, prophages, transposons and insertion sequences. The bacterial genomes therefore become pocked by small chromosomal replacements from other lineages. The frequency of such recombinational replacements in bacterial species may vary from very low to very high relative to mutational rates.

Horizontal genetic exchange can account for increased diversity in bacterial populations, but may also allow a successful allele to spread horizontally in a population replacing other variants and thereby reduce diversity at a locus.

Differences in the ratio of genetic change caused by recombination relative to *de novo* mutations leads to a spectrum of population structures from the extremes of clonal, with no recombination to non-clonal or panmictic where recombinational exchanges are sufficiently frequent to randomise the alleles in the population.

Most bacterial species probably occupy the middle ground.

In the study of the population biology of bacteria, important considerations are the assembly of representative collections of the organisms and accurate and appropriate characterisation. Unfortunately most existing culture collections are rarely representative of biological or natural populations as they frequently concentrate only on the most virulent, medically important isolates. As many bacterial pathogens do not rely on the generation of a pathological condition for transmission or their long-term survival, but cause disease accidentally or opportunistically, bias is introduced into the collections. These problems can be addressed by sampling strategies that take into account the biology of the particular pathogen, usually necessitating the establishment of structured sampling programmes¹²³. The strategy for obtaining an appropriate sample of the bacterial population is also species specific, for an obligate human pathogen, a collection of disease causing isolates from global sources is likely to sample more of the natural population of that organism when compared to a pathogen that is normally a commensal, such as GBS. In the latter case, a representative sample of the entire population must mainly comprise isolates from healthy carriers. It may also need to take into account other sources of that organism, such as other non-human species or the environment.

Serological typing has been a popular method of characterising bacterial pathogens, including GBS for many years. However, as the entities detected by serological tests are usually located on the surface of the cell, the corresponding genes are likely to be under strong diversifying selective pressure by the host immune system and evolve rapidly. Only rarely will such markers provide

reliable information on the phylogenetic history of a species and may give highly misleading information about mutational rates and recombination. In the study of bacterial population structure, it is therefore preferable to utilise genetic variation that is neutral. Housekeeping genes, such as those involved in central metabolism, are suitable in this respect as they are responsible for essential biochemical processes, where functional constraint is likely to be high¹²⁴.

Prior to the introduction of automated DNA sequence determination, MLEE was the method of choice for analysis of bacterial population structure. This is an indirect method in which electrophoretic mobility of enzymes infers genetic variation in housekeeping genes. The disadvantages of MLEE compared with sequence based methods, other than technical difficulty, are that the only genetic changes that are detected are those which alter electrophoretic mobility and that enzymes with the same electrophoretic pattern could have different sequences¹²⁵.

1.2.ii *Multilocus Sequence Typing:*

Multilocus sequence typing (MLST) was proposed in 1998 as an approach, which could provide accurate data, appropriate for the epidemiological investigation of bacterial pathogens and which could reflect evolutionary biology¹²⁴. MLST is a sequence based typing method, which was developed using the principles of MLEE. Genetic variation in housekeeping genes continues to be the focus of study, but nucleotide sequence determination rather than electrophoretic mobility, identifies this variation. The advantages of

nucleotide sequencing include generic methodology and ease of comparison electronically. Automation is a realistic possibility^{121,124}.

The design of an effective multilocus sequence typing system is dependant on several factors. The assembly of a collection of isolates is critical and at least for the initial evaluation should be from diverse sources representing the ecology of the organism (as described above). Other factors include, the choice of genetic loci to be characterised, the design of primers for gene amplification and nucleotide sequence determination and optimisation of amplification reactions¹²⁵.

MLST can be used to provide access to data using the internet through the creation of large databases¹²⁶ which can be accessed at <http://www.mlst.net>. These databases, in addition to being virtual isolate collections, contain epidemiological and clinical information and other typing results, such as serotype.

1.2.ii.a Analysis of MLST data. The clonal complex, utilised in the analysis of MLST data comprises genetically related but not identical, bacteria¹²⁵. Many datasets will contain large numbers of allelic profiles or sequence types (STs), but analysis reveals that some are numerically more significant than others. Using techniques such as BURST (START version 1.05, <http://www.mlst.net>) or split decomposition¹²⁷, it can be seen that these prevalent allelic profiles are frequently centrally located and have numerous relatives derived from them by a limited number of genetic events. These central

genotypes form the basis of the naming system of the clonal complexes, e.g. ST-23 complex of GBS has ST-23 as its central genotype. Organisation of data into clonal complexes allows easier epidemiological analyses¹²⁵.

Nucleotide sequence data from multiple housekeeping genes in an appropriately sampled population can be used in a variety of analyses. The simplest is to establish the alleles present at each locus and to use a clustering algorithm to determine the relationships between strains from the matrix of pairwise differences between their allelic profiles. While this is effective in establishing that isolates are identical or closely related, the approach will not provide much information about the relationships between more distantly related isolates, unless the population structure is strictly clonal.

Additional phylogenetic information can be recovered if the nucleotide sequences themselves are analysed. Measures of genetic change, which take into account the actual number of base changes between sequences rather than just whether the sequences are the same or different are more informative. One of the most common of these measures is the nucleotide diversity (or π) that represents the average number of nucleotide differences per site between two sequences. The number of variable nucleotide sites in a sample of sequences is denoted as S (or segregating sites). In order to be comparable across datasets, S/L , where L is the sequence length is a better measure of genetic diversity. Another indication of molecular genetic variation in populations is the average number of pairwise differences between sequences (Π).

Traditionally, phylogenetic data are depicted as a tree like structure. A tree is a mathematical structure, which is used to model the actual evolutionary history of a group of sequences. A phylogenetic tree is composed of branches and nodes. Branches connect nodes and a node is the point at which 2 or more branches diverge. Nodes can be internal, corresponding to the hypothetical last ancestor and external (terminal), which correspond to the sequences from which the tree is constructed ¹²⁸.

Molecular phylogenetic trees are usually drawn with proportional branch lengths; that is the lengths of the branches correspond to the amount of evolution between the two nodes they connect. The longer the branch, the more divergent are the sequences attached to them.

Molecular trees are based on multiple sequence alignments and are commonly constructed by the method known as “progressive sequence alignment”. This method builds up alignment in stepwise fashion, starting with the most similar sequences and progressively adding the more dissimilar ones. A crude guide tree is formed which is then built upon ¹²⁸.

The methods for calculating trees fall into 2 general categories. Distance-based matrix methods, also known as clustering or algorithmic methods (e.g. Unweighted pair group method with arithmetic means (UPGMA), neighbour-joining) and discrete data methods, known as tree-searching methods (maximum-likelihood, parsimony). Distance methods are simple and are rapidly calculated. The “distance”, the percentage sequence difference, is calculated for

all pairwise combinations of sequences and the distances are assembled into a tree. Discrete data methods examine each column of the alignment separately and look for the tree that best accommodates all of this data. For these reasons, the discrete data analyses contain more information ^{128,129}.

Once the tree has been generated, its accuracy can be tested. The simplest test of phylogenetic accuracy is the bootstrap. Bootstrapping essentially tests whether the dataset is supporting the generated tree. Random sub-samples of the dataset are taken, trees are built from these and the frequency with which the various parts of the tree are reproduced in each of the sub-samples is calculated. The bootstrap value given to a group is the percentage of occasions where that group is found in sub-sample trees. Bootstrap values of 70% or higher are thought to indicate reliable groupings ¹²⁸.

There are well-described pitfalls identified in phylogenetic tree construction. One of these is a phenomenon described as “long branch attraction” ¹²⁸. This is the tendency of highly divergent sequences to group together in a tree regardless of their true relationships. One effect of this is that bootstrap values all over the tree disintegrate. Removal of divergent sequences may reveal more accurately the relationships of the remaining strains in the collection.

Displaying evolutionary relationships as phylogenetic trees implies that evolution is a branching or tree-like process. However, a set of data often contains a number of different or conflicting signals, which may not support a tree structure. To address this problem the method of split decomposition was

developed ¹²⁷. In contrast to methods like maximum parsimony, that reconstructs trees by optimising certain parameters, split decomposition is a transformation-based approach. The evolutionary data are transformed or more precisely canonically decomposed into a sum of weakly compatible splits and then represented by a splits graph. For some data this is a tree, whereas other data will give rise to a tree-like network that can be interpreted as possible evidence for different and conflicting phylogenies. Furthermore, as split decomposition does not attempt to force data onto a tree it can provide a good indication of how tree like given data are. This is depicted graphically in Figures 1.1 and 1.2 where the tree like structure imposed on the data by the algorithms of UPGMA is then depicted as a network relationship of STs when analysed using split decomposition.

Figure 1.1: UPGMA tree (with bootstrap values) generated using concatenated sequence data for 5 STs of GBS.

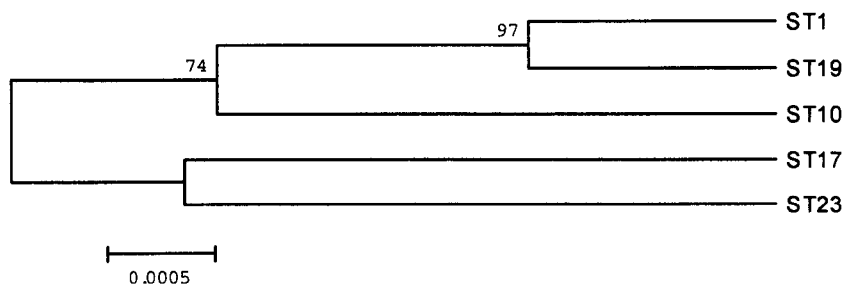
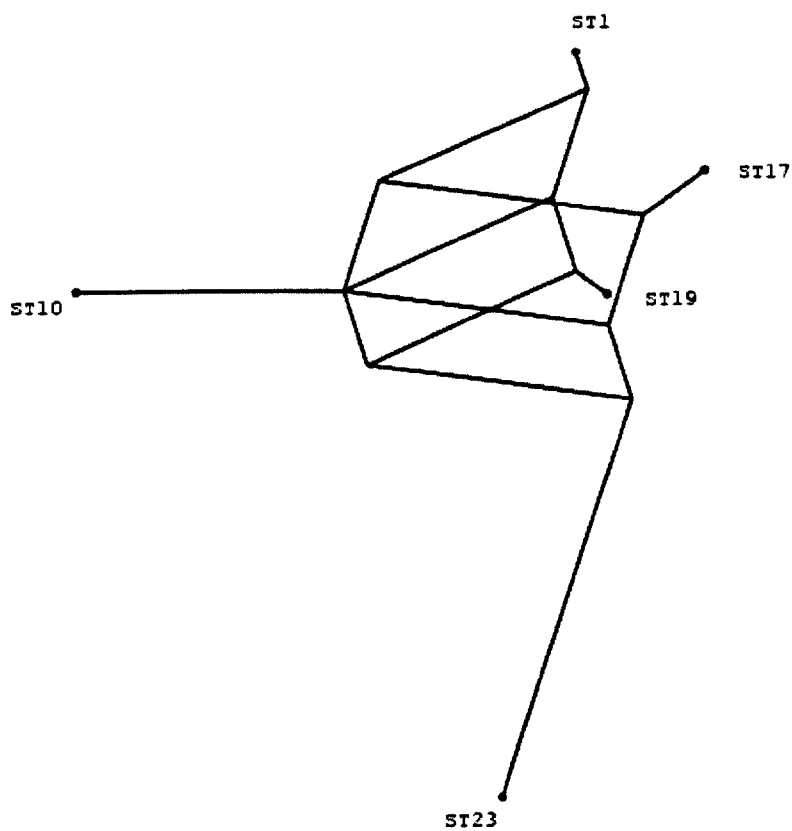


Figure 1.2: Tree generated using concatenated sequence data by split decomposition of 5 STs of GBS showing a network relationship of strains.



1.2.iii The evaluation of a typing system.

The discrimination of bacterial pathogens on the basis of genetic or phenotypic markers is imperative in descriptive epidemiology. To be able to investigate the diversity of organisms in defined environments and to describe the spread of particular strains of pathogen between hosts, it is essential to deploy a typing technique, which has the ability to distinguish between diverse strains of that organism.

Capsular serotyping is the technique that has been most commonly utilised and is the gold standard method for sub-typing of GBS. There is no standardised method for capsular serotyping therefore methodology varies between laboratories. The commonly used techniques rely upon the development of monoclonal antibodies against the 9 GBS capsular types and detection of agglutination using latex, capillary diffusion or other methods. Capsular serotyping has been popular because it is reasonably inexpensive and technically not demanding. Non-typable isolates, however, are fairly common in humans and much more common in bovine GBS. In addition, discrimination is thought to be less when compared with MLEE, RDP typing and PFGE, where isolates with the same capsule can be genotypically distinct.

In the evaluation of the efficiency a new typing system, typability, reproducibility and discrimination¹³⁰ are assessed. The typability of a method is the percentage of distinct bacterial strains, which can be assigned a positive typing marker. Reproducibility is the percentage of strains that give the same result on repeated testing. The discriminatory power is the ability of the method

to distinguish between unrelated strains. It is determined by the number of types defined by the test method and the relative frequencies of these types. It has been difficult to define discrimination using a single comparative statistic. Hunter et al¹³¹ have suggested the use of the Simpson's Index of Discrimination (D) as a single numerical index of discrimination, based upon Simpson's index of population diversity¹³². The index calculates the probability that two unrelated strains sampled from the same population will be placed in different typing groups.

1.3 AIMS OF THIS PROJECT:

The aims of this study are to investigate the population biology of an important human pathogen, GBS, and to determine whether hypervirulent clones of GBS (that is clones of GBS which are over represented in invasive disease rather than carriage and are therefore likely to be associated with virulence determinants) can be identified and correlated with disease in humans.

1. To develop a multilocus sequence typing system for GBS which could then be used to examine the population biology of GBS.
2. To validate the GBS MLST system on a global collection of human isolates isolated from carriage and invasive disease.
3. To investigate the population biology of GBS on assembled collections of GBS representing human carriage and disease in neonatal and adult groups from a single geographical area (UK).
4. A further aim was to examine bovine GBS and its relationship with human GBS.

Chapter 2: Materials and Methods:

Generic methods are described below. Additional methods specific to a particular study are included in the chapter describing that study.

2.1 MICROBIOLOGY METHODS:

2.1.i *Identification of GBS:*

Group B streptococci were cultured in the laboratory on Colombia agar plates containing 5% horse blood and identity confirmed as GBS according to standard methods. Incubation of plates was carried out overnight in a humidified atmosphere enriched with carbon-dioxide (5%). White or buff coloured glistening, mucoid colonies approximately 1-3 mm in diameter surrounded by a small zone of β -haemolysis were further identified. Catalase-negative, Gram-positive cocci in pairs or short chains were confirmed as GBS using the rapid latex agglutination test (Oxoid, Basingstoke, UK), which detects the Lancefield group B antigen.

2.1.ii *Lancefield group B antigen detection by latex agglutination:*

All reagents, antisera and controls were provided by Oxoid, Basingstoke, UK. A small glass test-tube was labelled for each organism to be tested and for a positive control. One hundred and fifty microlitres of extraction enzyme was added to each tube. Approximately five colonies were picked off and emulsified into the extraction enzyme with a flamed platinum loop. The tubes were incubated in a water bath at 37°C for 10- 15 mins. Antisera for Lancefield antigen B, D, and F were tested against each isolate. Agglutination confirmed the presence of the Lancefield B antigen.

2.1.iii *Assembly of freezer files of GBS isolates:*

Isolates of GBS for study were kept frozen at -80°C in Tryptone-soy broth with glycerol (10 %).

2.1.iv *Capsular serotyping methods:*

The capsular serotypes of the GBS isolates were determined using latex agglutination. The methods followed were based upon those of Slotved et al¹³³. Antisera to the nine GBS serotypes (IA, IB to VIII) were supplied by State Serum Institute, Denmark. Strains were cultured overnight on blood agar plates then inoculated into Todd-Hewitt broths (1ml) in sterile capped tubes. Tubes were incubated overnight at 37°C. On a glass slide 1µl of antisera was mixed with 5µl of broth suspension of organism. The slide was rocked for a maximum of 30 seconds. Agglutination indicated a positive reaction.

2.2 MULTILOCUS SEQUENCE TYPING METHODS:

2.2.i *Extraction of DNA from isolates:*

A kit method (Qiagen DNeasy kit) was followed. All reagents were supplied by Qiagen, Germany, other than those for enzymatic lysis buffer, which were supplied by Sigma, USA. Isolates were sub cultured from -80°C freezer vials to 5% horse blood agar plates and incubated overnight as described. Using a sterile loop, a single colony from the blood agar plates was streaked out onto a second blood agar plate and overnight incubation repeated.

Following incubation, approximately 5 colonies from the blood agar plate were used to inoculate 200µl Phosphate Buffered Solution in a sterile tube. Tubes

were centrifuged at $5,500 \times g$ for 10 min. The supernatant was poured off, being careful not to disturb the pellet.

The bacterial pellet was resuspended in 180 μ l enzymatic lysis buffer, containing 20 mM Tris-Cl (pH 8.0), 2mM Ethylenediaminetetraacetic acid (EDTA), 1.2% Triton® X-100 and 20 mg/ml lysozyme. The tubes were incubated in a water bath at 37°C for 30 minutes. Then 25 μ l Proteinase K and 200 μ l Buffer AL were added and incubation continued at 70°C in a water bath for 30 minutes. Ethanol 96% (vol./vol.) 200 μ l to precipitate the DNA was added to the sample and mixed by vortexing. This mixture was pipetted into the DNeasy mini columns (Qiagen) and centrifuged at $5,500 \times g$ for 1 minute. The flow through and collection tube were discarded and the column placed into a fresh collection tube. The column was then washed using ethanol-based buffers (AW1 and AW2, Qiagen). The DNA was eluted in sterile water and then stored frozen at -20°C.

2.2.ii *Polymerase chain reaction (PCR) methods:*

The methods utilised for PCR, DNA precipitation and sequencing were based upon those in use in the host laboratory and have been published previously¹³⁴. Fifty microlitre PCR reactions were set up in 96 well plates. Each well contained the following reagents:- Forward and reverse primers 1 μ l each (Table 5.1, 100 pmol/ μ l, supplied by MWG-biotech, Germany), buffer 5 μ l (MgCl₂ 15mM, pH 8.7, 10 \times concentrated, Qiagen, Germany), dNTPs 1 μ l (Deoxyribose nucleotide triphosphate, 10 mM, supplied by ABgene, UK), Taq polymerase 0.5 μ l (5 units/ μ l Qiagen, Germany), sterile water 39.5 μ l and DNA template 2 μ l. The PCR cycle consisted of 30 cycles with denaturation at 94°C, annealing at 55°C

and extension at 72°C. During development of the MLST system, the annealing temperature was varied between 45 and 65°C in certain experiments.

2.2.iii *Demonstration of PCR products on agarose gel:*

Agarose gels were used for confirmation of the presence of PCR amplicons. Gels of 1% agarose (Helena Biosciences) were made using a base of tris-borate EDTA buffer (TBE, 1×, Sigma, USA). Microwave heating dissolved the agarose. Ethidium bromide (10mg/ml Sigma, USA) was added to the molten gel to label DNA, with 1µl of ethidium bromide solution added to 50mls agarose. Combs were placed in the liquid gel to generate wells of 10µl. The gels were allowed to set at room temperature. Five microlitres of template, consisting of 2µl PCR product and 3µl dye-loading buffer (Sigma, USA), were added to each well. The 1kb DNA ladder (Promega) was used as a size marker (5µl per well) for PCR amplicons of 500-700 base-pairs. Gels were placed in an electrophoresis tank filled with TBE buffer (1×) and electrophoresis proceeded at 50-100 mV for 15-45 minutes. Gels were visualised using a UV camera and the Molecular Analyst programme (version 2.1.2, BioRad Laboratories,UK).

2.2.iv *Precipitation of PCR amplicons:*

All reagents were provided by Sigma, USA. Sixty microliters of 20% polyethylene glycol (PEG-8000) with 2.5M Sodium Chloride (NaCl) was added to each PCR reaction. Plates were then placed at room temperature for 30 minutes incubation. Centrifugation was then carried out for 60 minutes at 2,465 × g. The supernatant was then removed by spinning the plates upside down at 50 × g on paper towels. Two ethanol washes were then carried out with 150µl

ethanol 70% (vol./vol.) per well and centrifugation ($2,465 \times g$) for 20 minutes.

The precipitated DNA was then dried by removal of supernatant ($50 \times g$) and incubation at 37°C for 15 minutes. DNA was eluted by the addition of $10\mu\text{l}$ sterile water per well.

2.2.v *DNA nucleotide sequence determination:*

Nucleotide sequences were determined at least once on each DNA strand using internal nested primers (Table 5.1, concentration 1 picomols per μl , MWG, Germany) and ABI PRISM® BigDye™ Terminators v 3.0 Reaction Mix (Applied Biosystems, Foster City, USA) in accordance with the manufacturer's instructions. Sequencing reactions were carried out in 96-well plates, the forward reactions were carried out in the left hand 48-wells of the plate and the corresponding reverse reactions were carried out in the right hand 48 wells of the plate. The sequencing reactions were carried out on the DNA engine using a programme with 30 cycles of denaturation at 96°C , annealing at 50°C and extension at 60°C . Unincorporated dye terminators were removed by precipitation of the termination products with Sodium acetate (3M, pH 5.2) and ethanol 96% (vol./vol.), and the reaction products were separated and detected with an ABI PRISM® 3700 DNA Analyser (Applied Biosystems, Foster City, USA). Sequences were assembled from the resultant chromatograms with the STADEN suite of computer programs and edited to resolve any ambiguities¹³⁵. The STARS software (available on www.mlst.net¹³⁶) was used to reverse compliment and align sequence pairs.

2.2.vi *Adaptations of method for tube reactions:*

PCR and sequencing reactions were carried out in 200µl PCR tubes, during development and when small numbers of reactions were carried out. The reactions were set up in the same way as those described above for the wells of a 96-well plate. Centrifugation was carried out at $15,700 \times g$ for similar time span as that described above for the plate centrifuge. Supernatant was removed by pipette.

2.2.vii *Allele and Sequence Type (ST) assignment:*

For each locus, every different sequence was assigned a distinct allele number in order of identification; these are internal fragments of the gene, which contain an exact number of codons. Any change in the nucleotide sequence, whether or not the amino-acid sequence is altered, was defined as a new allele. Each isolate was therefore designated a seven-integer number, constituting its allelic profile. Isolates with the same allelic profile were assigned to the same ST, which are numbered in the order of their identification (ST-1, ST-2 etc). The data were been deposited in a database accessible on the Internet at <http://sagalactiae.mlst.net>¹²⁶.

2.3 COMPUTATIONAL ANALYSES:

2.3.i *Analyses based upon allelic profile:*

The START series of programmes were used to group STs into lineages or clonal complexes using BURST (START version 1.05, <http://www.mlst.net>¹³⁶). The members of a BURST lineage were defined as groups of two or more independent isolates where each isolate shared identical alleles at six or more

loci with at least one other member of the group. UPGMA trees could also be drawn from allelic profiles using the START software.

2.3.ii *Analyses based upon nucleotide sequences:*

Nucleotide sequence data were used to analyse the population structure of collections of strains. Each strain was represented by a concatenated sequence. That is, the sequences of the seven housekeeping genes were run together, so that the unit of study was one long length of 3456 base-pairs, rather than seven separate sequences of approximately 500 base-pairs.

Several software programmes were used to analyse nucleotide data from concatenated sequences. Measures of genetic diversity and neutrality of mutations were studied using DnaSP (DNA sequence polymorphism, version 3.99, www.ub.es/dnasp¹³⁷). Phylogenetic trees were drawn using the algorithms of UPGMA, neighbour-joining and maximum-parsimony in the programmes contained in MEGA (Molecular Evolutionary Genetic Analysis, version 2.1, www.megasoftware.net¹³⁸). The split decomposition method was utilised and resultant trees drawn by the SplitsTree programme (version 3.2, <http://bibiserv.techfak.uni-bielefeld.de/splits>¹²⁷). The significance of the tree structures was calculated and Bootstrap values allocated to the branches using either MEGA or SplitsTree.

A clonal complex was defined as closely related STs, which are grouped together on a phylogenetic tree¹²⁵. The clonal complex was named after the ST identified by BURST as the central sequence e.g. ST-19 complex, or if BURST

was not able to define the ancestor, then the most numerous ST became the name of the complex e.g. ST-6 complex.

Diversity within and between clonal complexes was examined using concatenated sequences. The analyses (carried out using MEGA) included the number of segregating sites (S), the average number of pairwise nucleotide differences (Π) and nucleotide diversity as measured by π . The number of pairwise differences (Π) was estimated from the equation:

Figure 2.1: Calculation of average number of pairwise differences.

$$\Pi = \frac{1}{n(n-1)/2} \sum_{i < j} \Pi_{ij}$$

Where n is the number of sequences and $n(n-1)/2$ is the number of pairwise comparisons and Π_{ij} is the difference between the i th and j th sequences. Π can then be divided by L (the length of sequences, 3456 bp), where Π/L gives the value of π ¹²⁹.

The Simpson's index of diversity (D) was used to assess diversity between carried and invasive populations of GBS as it also takes into account the frequency of allelic profiles represented in a collection. The index was calculated according to the equation:

Figure 2.2: Simpson's index of diversity.

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^s n_j (n_j - 1)$$

Where N is the total number of strains in the sample population, s is the total number of strains described and n_j is the number of strains belonging to the j th type.

The 95% confidence intervals (CI) were calculated using the variance, σ^2 , given by the equation:

Figure 2.3: Calculation of variance of a population.

$$\sigma^2 = 4/n \left[\sum \pi_j^3 - \left(\sum \pi_j^2 \right)^2 \right]$$

where π_j is the frequency n_j / n , n_j is the number of strains belonging to the j th type, and n is the total number of strains in the sample population. An approximation of the 95% CI is then given by the equation:

Figure 2.4: Calculation of CI around the index.

$$CI = [D + 2 \sqrt{\sigma^2}, D - 2 \sqrt{\sigma^2}]$$

2.4 EPIDEMIOLOGICAL ANALYSES:

Epi Info 2002 software from the Centers for Disease Control and Prevention (CDC, www.cdc.gov) was used to calculate odds ratios, 95% CI and levels of significance .

An empirical odds ratio (OR) was calculated to compare the probability of invasive disease due to individual STs, clonal complexes or serotypes. The OR was calculated by reference to all other serotypes or clones using published methods¹³⁹ as follows: $OR = ad/bc$, where a is the number of invasive A STs or serotypes, b is the number of carried A STs or serotypes, c is the number of invasive non- A STs or serotypes and d is the number of carried non-A STs or serotypes.

Yates corrected test of significance was used to calculate p-values, except where the value of a cell was less than 5, in which case the Fisher exact test was used.

2.5 ASSESSMENT OF DISCRIMINATION OF GBS MLST:

Quality control of MLST was achieved in several ways. Laboratory methods were fastidious. A negative control (well containing sterile water in place of DNA) and a positive control (strain with a known allelic profile) were included on each 96- well plate. A random selection of PCR and sequencing reactions were repeated to confirm allelic identity.

Simpson's Index of Diversity (D) (Figure 2.2) was used to determine the discrimination of the MLST system and the capsular serotyping method for GBS. The index calculates the probability that two unrelated strains sampled from the same population will be placed in different typing groups. Ninety-five percent CI were calculated from the equations in Figures 2.3, 2.4.

Chapter 3: Invasive GBS disease in Oxford, UK.

3.1 AIMS:

1. Development of a collection of invasive neonatal and adult isolates of GBS for further study.
2. Assessment of the incidence of invasive GBS disease in population age-groups in Oxfordshire.

3.2 INTRODUCTION:

The incidence of invasive GBS infection has been studied in some detail in North America, and less so in the UK. A recent national surveillance project took place in the UK and found that 0.8/1000 of neonates in England and Wales is affected by invasive neonatal GBS disease⁶⁰. Adults are known to account for more than half of all invasive GBS cases⁹¹, and it has been suggested that GBS infection is increasing in adults, particularly those with medical disorders, pregnant women and the elderly⁹⁵. There have been few studies of adults in the UK with invasive GBS infection.

3.3 MATERIALS AND METHODS:

3.3.i Enhanced surveillance of hospitals in the Oxford region:

GBS isolates from normally sterile sites were collected for a retrospective period from 1995 to 2000 and prospectively from March 2000 to September 2003.

3.3.i.a Retrospective study, John Radcliffe and Horton Hospitals, Microbiology Laboratories. The retrospective collection of isolates was

assembled by interrogation of the laboratory computer databases of freezer files. Isolates were retrieved from freezer vials (Tryptone-soy broth with glycerol) being stored at -80°C.

3.3.i.b Prospective study: John Radcliffe Hospital and Hospitals in the Oxford region. The microbiology laboratory at the John Radcliffe hospital (JRH) was the host laboratory. Medical microbiologists and biomedical scientists were made aware of the study. In addition, weekly computer alerts were printed out listing all the group B streptococci isolated from normally sterile sites over that week.

Consultant microbiologists from the hospitals within the Oxford region were informed by telephone, by post and by means of a presentation at the regional microbiology meeting “the bug club” of the group B streptococcal research in progress in Oxford. The microbiologists were asked to send every group B streptococcal organism isolated in their laboratory to the research laboratory at Oxford. They were asked to fill in a brief form to accompany the isolates (Figure 3.1). The microbiology departments taking part were from the hospitals in Banbury, Bedford, Kettering, Luton, Milton Keynes, Northampton, Reading, Aylesbury, Slough and High Wycombe. The participating departments were informed of results and encouraged to continue taking part by means of a biannual newsletter (Figure 3.2) and by regular presentations at the “bug club”.


Figure 3.1: GBS invasive infection form to accompany isolates sent to the study laboratory.

<p>GROUP B STREPTOCOCCAL STUDY Dr Nicola Jones Research Lab John Radcliffe Hospital level 7 Oxford OX3 9DU</p> <p>Group B streptococcus has been isolated:-</p> <ul style="list-style-type: none">• Specimen type: Blood/ CSF/ Joint asp/ Bone/ Other (please state)• Date of specimen• Hospital where GBS was isolated• Micro lab number• Name of patient or initials• Patient's hospital number• Patient's date of birth• Brief clinical details <p>Any comments:</p>

Figure 3.2: GBS newsletter circulated to Microbiology Consortium six monthly.

1st February 2002

GBS NEWSLETTER
DEPARTMENT OF MICROBIOLOGY
JOHN RADCLIFFE HOSPITAL
OXFORD OX3 9DU



GBS CARRIAGE STUDY
HORTON HOSPITAL

Action Research has funded a carriage study at the Horton Hospital and JR Hospital Maternity Units. This study has now started and is expected to run for a further 6 months.

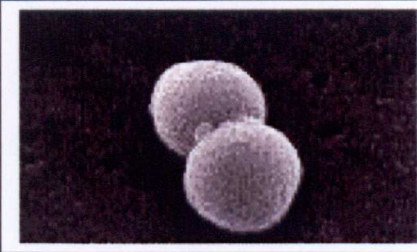
GROUP B STREPTOCOCCAL STUDY
– **Invasive disease**

Thank you for participating in this study and sending isolates of GBS from normally sterile sites, including blood, CSF and joint fluids.

Over the page is a breakdown of the figures since 1st April 2001 until 31st January 2002.

The study is on going, so please continue to send any isolates to the lab in.

This project is funded by the MRC.



GBS READING.....

National Interest: “Confirmed **group B streptococcus** infection: the tip of the iceberg”. Estimated neonatal GBS infection rates from St George’s Hospital, London are 2.6/1000 live births. Bedford Russell A et al. Arch Dis Child Fetal Neonatal Ed 2001; 84: F 140.

Microbiology: “Use of GBS media for rapid detection of Group B streptococcus in vaginal and rectal swabs from women in labour”. GBS broth and plates (Islam – type media) were compared to CDC recommended techniques for isolation of GBS. Further study is needed. Votava M et al. Eur J Clin Microbiol Infect Dis 2001; 20: 120-22.

Carriage studies: “Group B streptococcus colonisation in male and non- pregnant female university students”. One third of college students were carrying GBS in GU/ rectal sites. Carriage persisted for 6 months in the vast majority. Bliss SJ et al. Clin Infect Dis 2002; 34:184-90.

Prevention: “Group B streptococcal disease. From trials and tribulations to triumph and trepidation”. Review article of preventative strategies adopted in USA and their results. Potential and actual adverse effects associated with such strategies are also covered. Schuchat A. Clin Infect Dis 2001; 33: 751-6.

From Dr Nicola Jones (specialist registrar microbiology, Oxford)

3.3.ii *Microbiological handling of isolates:*

Isolates received in the laboratory were plated onto Colombia agar with 5% horse blood then incubated overnight at 37°C in a CO₂ enriched (5%) environment. Colonies were then confirmed as GBS according to the methods described in Chapter 2.

3.3.iii *Database of clinical information:*

A Microsoft Access database was developed. Details listed were the patient's initials, date of birth, specimen type, date of collection of isolates, brief details of diagnosis and referring hospital.

3.3.iv *Calculation of incidence figures:*

Incidence figures were calculated using data from the hospitals within Oxfordshire (the JRH, Oxford and Horton Hospital, Banbury) only. Data covering three-years from September 2000 to September 2003, within the prospective study period, were used. The 2001 census (<http://www.statistics.gov.uk>) was used as the source of population numbers in age groups in Oxfordshire. Adult incidence was expressed as cases per 100,000 population per year. Neonatal incidence figures for invasive GBS disease were expressed as figures per 1000 live births. Birth rates were supplied by the Maternity Units, John Radcliffe and Horton Hospitals.

3.4 RESULTS:

The enhanced surveillance project resulted in a collection of 299 cases of invasive GBS (Table 3.1). Seventy-six cases were identified by the retrospective study (1995-2000), from freezer files of organisms kept at the John Radcliffe (66 cases) and Horton Hospitals (10 cases). The remainder (223 cases) were identified prospectively from the Hospitals within the Oxford region.

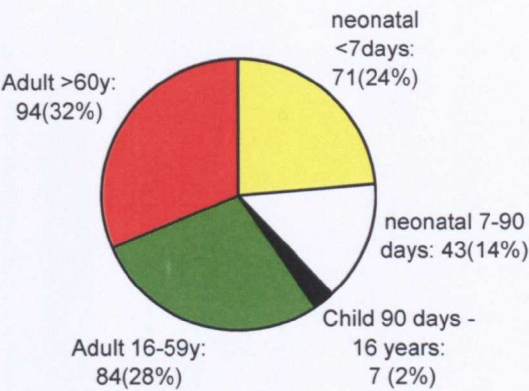
Table 3.1: GBS invasive disease: numbers of cases according to hospital and age group.

Hospital	Neonate <7 days	Neonate 7-90 days	Child 90 days - 16 years	Adult 16-59 years	Adult >60 years	TOTALS
Oxford	27	15	6	46 (20)*	46 (11)**	140
Aylesbury	5	2	0	2 (2)	1 (0)	10
Banbury	8	2	0	1 (0)	4 (2)	15
Bedford	6	3	1	4 (4)	6 (2)	20
High Wycombe	5	0	0	8 (6)	7 (2)	20
Kettering	3	2	0	4 (0)	7 (1)	16
Luton	4	5	0	4 (3)	4 (1)	17
Milton Keynes	3	1	0	2 (1)	0 (0)	6
Northampton	1	1	0	2 (2)	0 (0)	4
Reading	6	10	0	3 (2)	10 (4)	29
Slough	3	2	0	8 (2)	9 (4)	22
TOTALS	71	43	7	84 (42)	94 (23)	299

Numbers in parentheses are cases of invasive GBS disease in *adult females 16-44 years and ** adults over 80 years.

The distribution of invasive GBS infection according to age group for the entire population is illustrated graphically in Figure 3.3.

Figure 3.3: Invasive disease due to GBS according to age group (n=299).



It can be seen that approximately one-third of cases were found in neonates, with early-onset disease accounting for slightly more than late-onset. Sixty percent of infections affected adults, with roughly equal numbers being less than 60 years and over 60 years of age. The age distribution at onset of infection is shown for neonates in Figure 3.4 a and b.

Clinical diagnosis is indicated in Figure 3.5 for the Oxford population.

Diagnoses were determined for all neonatal cases and is shown in Figures 3.6 a and b for early and late-onset neonatal disease.

Figure 3.4: Age at onset for cases of early onset (n=71), (a) and late (n=43) (b) onset GBS neonatal disease.

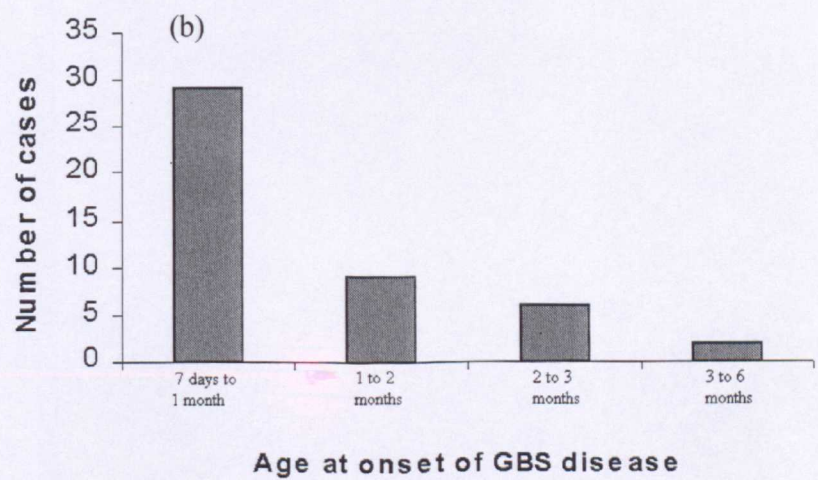
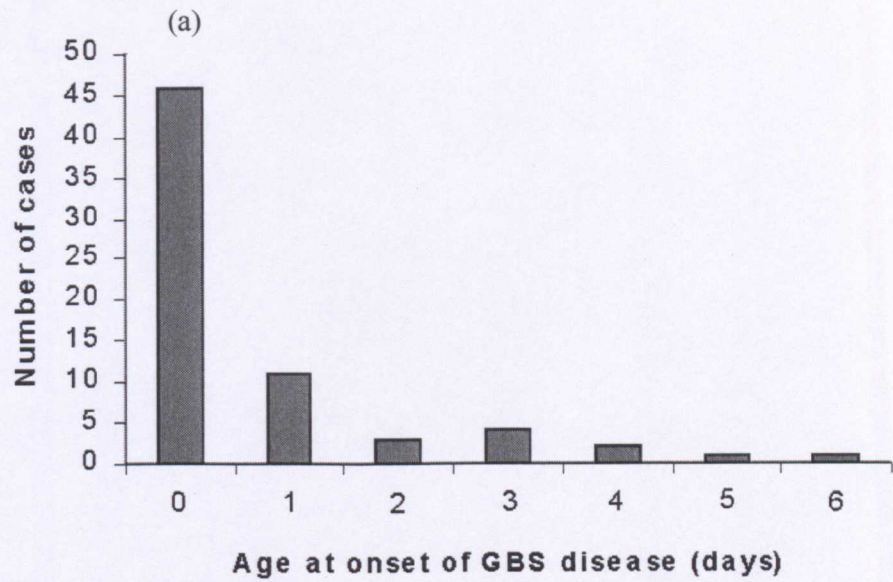


Figure 3.5: Diagnoses associated with invasive GBS disease in all age groups (n=140). Figures are number (%); 25 had more than one diagnosis.

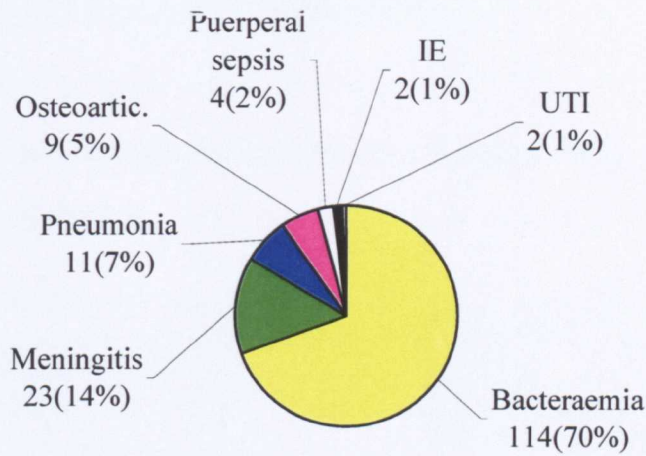
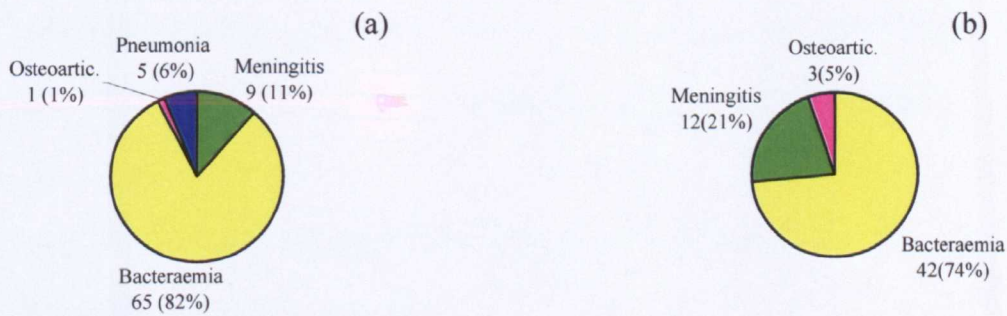


Figure 3.6: Clinical diagnosis of neonatal invasive GBS for early onset (n=71) (a) and late onset (n=43) (b) infection.



Figures are number (%). More than one diagnosis was recorded for 9 early and 14 late cases.

The calculated incidences (Table 3.2) of GBS invasive disease affecting adults in the Oxfordshire region in different age groups showed the highest incidence to be in adults over 60 years (6.1/ 100,000/ year).

Table 3.2: Incidence of adult GBS infection, according to age group from September 2000-2003.

Age	No. cases	Population *	Incidence ⁺
Adult 16-59 years	22	379,065	1.9
Adult >60 years	21	115,349	6.1
Overall	43	494,414	2.9

* Population of Oxfordshire from 2001 census (<http://www.statistics.gov.uk>)

⁺ incidence: cases of invasive GBS disease per 100,000 population in that age group/ year.

The birth cohort for the period September 2000-2003 at the JRH was 22,275 live births (Table 3.3). The calculated incidence of neonatal GBS infection was 0.94/1000 live births overall, with 0.63/1000, early-onset and 0.31/1000, late-onset. Using the birth cohort as the number of pregnant women, the incidence of cases of GBS infection in pregnant women was calculated as 18.0/100,000.

Table 3.3: Incidence of neonatal GBS infection, early and late onset in Oxfordshire September 2000-2003.

Neonatal GBS	No. cases	Birth cohort	Incidence*
Early-onset	14	22275	0.63
Late-onset	7	22275	0.31
Both	21	22275	0.94

*Incidence, cases of invasive GBS disease per 1000 live births

Case record review was carried out for all neonates with GBS disease admitted to the JRH. Thirty-four of 42 case records could be retrieved. Maternal risk factors for neonatal GBS disease were identified in 13 of 25 cases of early onset infection. The occurrence of risk factors is shown in Table 3.4. Twenty-three complications were seen in 34 cases of neonatal GBS infection, which are listed (Table 3.5). Four cases (11.8%) proved to be fatal. Surface swabs were taken from 17 neonates with early onset GBS disease. GBS was isolated most frequently from ear and throat cultures (Figure 3.7).

Table 3.4: Risk factors identified in the mothers of 25 cases of early onset neonatal GBS disease.

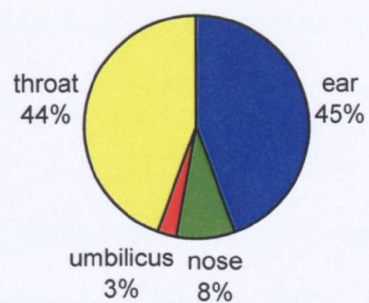
Risk factor:	Number of cases (%)
No identified risk factor	12 (48)
Prolonged rupture of membranes	5 (20)
Prematurity (<37 weeks)	4 (16)
Fever (>38°C)	3 (12)
PROM, prematurity	1 (4)
PROM, fever	2 (8)
PROM, fever, prematurity	1 (4)

Abbreviations: PROM, prolonged rupture of membranes >18 hours.

Table 3.5: Clinical manifestations recorded in 34 cases of neonatal GBS disease (early and late onset).

Clinical manifestation:	Number of cases
Respiratory failure	6
Seizures	5
Coagulopathy	3
Severe liver impairment	2
Acute renal failure	1
Hydrocephalus	1
Intraventricular haemorrhage	1
Fatal outcome	4

Figure 3.7: Surface swab cultures from neonates (n=17) with early onset GBS infection. Positive results at each site are recorded as %.



3.5 DISCUSSION:

Almost 300 isolates from cases of invasive GBS disease were collected over eight years, both retrospectively and prospectively. Incidence rates of GBS invasive infection were calculated for Oxfordshire using 2001 census figures and live birth rates.

3.5.i Neonatal GBS incidence rates:

The overall incidence of neonatal GBS infection in Oxfordshire was 0.94/1000 live births, of which 67% were cases of early-onset infection. These incidence rates are similar to those found in previous studies in the UK. In particular the incidence of early-onset GBS infection was found to be 0.5/1000 in 1998 in Oxford⁶⁹, 0.95/1000 in 1998 in Sunderland⁷⁰ and 0.5/1000 in the UK national surveillance study⁶⁰. The finding that approximately two-thirds of cases are early in onset is consistent with the results of other studies⁶⁰.

3.5.ii Adult GBS incidence rates:

Incidence of invasive GBS infection has not been well studied. Most of the available data comes from North America, where incidence rates of 4.1 to 7.2/100,000 have been described⁹¹. In a Canadian study, 41/100,000 pregnant women were diagnosed with invasive GBS infection compared with 4.1/100,000 in non-pregnant women⁹⁴. An investigation of people over the age of 65 years gave incidence rates of 17.5/100,000 of GBS invasive infection in those over 65 years living in the community, compared with 72.3/100,000 in those living in nursing homes⁹⁵. The incidence rates in Oxfordshire are somewhat lower than

these figures, with 2.9/100,000 people over the age of 60 years having invasive GBS infection per year. The ratio of approximately half the infections occurring in the age group under 60 years and half occurring after 65 years, is however, maintained.

Age distribution of cases shows that approximately one-third of cases of invasive GBS occur in neonates and the remainder in adults. Almost 50% of early onset neonatal cases occur in the first 24 hours. This is consistent with the findings of other studies⁶⁹. Clinical diagnoses associated with invasive GBS disease were predominantly bacteraemia with no focus and then meningitis. This was found when the entire population was studied and was also the case when neonates were studied in isolation.

Case record review for 34 neonates with GBS disease admitted to the JRH showed that maternal risk factors were not present in approximately 50% of cases. Complications of GBS disease occurred commonly in neonates, with respiratory failure and seizures being most prevalent. The neonatal mortality rate was 11%, which approximates with national published figures⁶⁰. Surface swabs taken from neonates with early onset infection showed that GBS was cultured most frequently from ear and throat sites, which has been previously demonstrated¹⁴⁰.

In conclusion, a large collection of GBS isolated from cases of invasive infection in all age groups has been assembled for further study using MLST. Incidence rates of infection have been calculated. Age distribution and diagnoses

associated with infection have been described. More detailed review of neonatal case records has given some information about risk factors for infection, complications of infection and mortality rates.

Chapter 4: Asymptomatic Carriage of GBS in Pregnant Women from Oxford, UK.

4.1 AIMS:

1. To undertake a carriage study in Oxfordshire of GBS carriage rates in pregnant women.
2. To determine risk factors for carriage.
3. To assemble a collection of carried isolates of GBS from pregnant women for molecular epidemiological study.

4.2 INTRODUCTION:

GBS is the leading cause of neonatal sepsis in many parts of the world including the UK⁶⁵. Administration of IAP to the GBS colonised woman is an effective strategy for the prevention of early-onset neonatal sepsis⁶⁷. The Royal College of Obstetricians (<http://www.rcog.org.uk>), and the Health Protection Agency (formerly PHLS) (<http://www.hpa.org.uk>) have published draft and interim guidelines for the prevention of neonatal GBS infection in the UK that recommend the identification of risk factors¹⁰⁸ in the mother to guide administration of IAP. The CDC have recommended universal screening in the USA to identify GBS carriers who will then receive IAP⁶⁶.

The development of national guidelines for prevention of neonatal GBS has been hampered in the UK by a lack of epidemiological data. The investigations that have been carried out have given different results. A study carried out in London

in 1987 identified a carriage rate of GBS of 28% in pregnant women³⁵, whereas results a study in North Wales (2001) detected a much lower rate of 4.3%³⁴. An investigation carried out in the Republic of Ireland (1998) was more akin to the London study, showing a 25.6% carriage rate³⁶.

4.3 MATERIALS AND METHODS:

A prospective carriage study was carried out in the Oxford area of the UK over a 2-year period (2001-3). Pregnant women attending antenatal care at hospital and community units attached to the JRH, Oxford, and at the Horton Hospital, Banbury were recruited. A single vagino-rectal swab for culture of GBS was taken from participants. Questionnaires (Figure 4.1 and Figure 4.2) were used to gather demographic and epidemiological data about the pregnancies and outcomes for women attending the Horton Hospital. No data were collected from women attending the JRH other than GBS carriage status. Local policy was followed to guide the administration of IAP. The Oxfordshire Ethics Committee granted ethical approval (C00.035, Appendix 2).

Figure 4.1: Questionnaire for recruited pregnant woman to complete.

PREVENTING INFECTIONS IN THE NEWBORN BABY THE HORTON HOSPITAL GBS CARRIAGE STUDY confidential questionnaire for patient																						
<p><i>Please tick Yes/No boxes and fill in the large boxes.</i></p>																						
1. How many weeks pregnant are you?	<input style="width: 50px; height: 30px; border: 1px solid black;" type="text"/>																					
2. Have you ever been Pregnant before?	Yes <input style="width: 30px; height: 20px; border: 1px solid black;" type="checkbox"/> No <input style="width: 30px; height: 20px; border: 1px solid black;" type="checkbox"/>																					
If Yes, How many times? <input style="width: 50px; height: 30px; border: 1px solid black;" type="text"/>																						
3. Have you taken antibiotics in the last three months?	Yes <input style="width: 30px; height: 20px; border: 1px solid black;" type="checkbox"/> No <input style="width: 30px; height: 20px; border: 1px solid black;" type="checkbox"/>																					
If yes, can you tell us what they were _____ and what they were for? _____																						
4. Which ethnic group do you consider yourself to belong to?																						
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6. What job do you/ did you do most recently? _____																						
7. If you are married or living with someone what job does your partner do? _____																						
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<p>Thank you very much for completing this questionnaire. Please hand it to the midwife as soon as possible. Any other comments?</p>																						

Figure 4.2: Questionnaire for Midwife in attendance to complete.

PREVENTING INFECTIONS IN THE NEWBORN BABY
THE HORTON HOSPITAL GBS CARRIAGE STUDY
Confidential Questionnaire for Midwife to complete

To be completed after the birth of the baby.

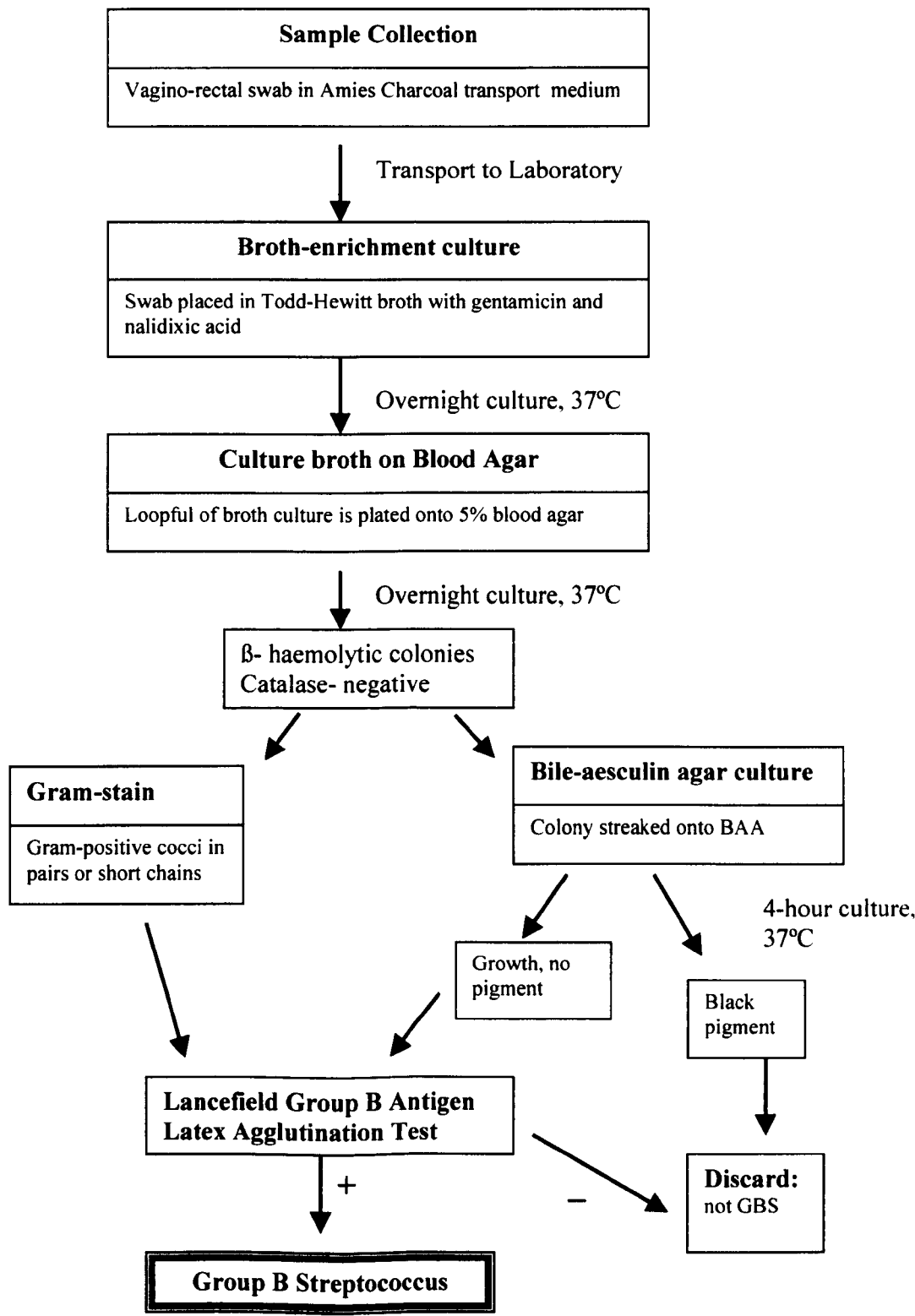
Please tick Yes/ No boxes

Patient's name	
DOB	
Hosp no	
Address	
	Or patient's sticky

1. Please fill in details for the baby: Male ☐ Female ☐ Weight (g) _____
2. Date of delivery/...../.....
3. Has GBS been isolated from the mother during pregnancy? No ☐
Yes ☐
If Yes, please ring site where GBS was isolated urine/ vagina/ blood/ placenta
4. Method of delivery: Spontaneous vaginal ☐
Ventouse extraction ☐
Forceps ☐
Caesarean section- emergency ☐
- elective ☐
Other _____
5. Did complications develop in the mother? No ☐ go to Q6
(Only during labour) Yes ☐
If yes, please give details: ☐ Fever > 38 degC
- Prolonged rupture of membranes >18 hours
- Premature delivery (< 37 weeks), at _____ weeks
Other _____
6. Were antibiotics given to the mother during labour? No ☐
Yes ☐
If yes which antibiotic..... PO ☐
IV ☐
Given > 4 hours ☐ or less than 4 hours ☐ prior to delivery

Women were recruited in the antenatal clinic, and a single vagino-rectal swab (a sweep with a swab from low vagina down the perineum to just inside the rectum) was then taken when the woman presented to the hospital in labour. The swabs were handled in the research microbiology laboratory at the JRH according to an algorithm (Figure 4.3) based upon standard methods⁶⁶. Swabs were cultured overnight at 37°C in 20 ml vials of Todd-Hewitt Broth containing gentamicin 8mg/L and nalidixic acid 15 mg/L⁶⁶ then plated out onto Columbia agar with 5% horse-blood for overnight culture in CO₂ at 37°C. β-haemolytic colonies, which were negative with catalase reagent, were further investigated by Gram-staining and culture on BAA¹⁴¹. Colonies growing on BAA without black pigment production were confirmed as GBS by Gram-staining and detection of Lancefield group B antigen by latex agglutination (Oxoid, Basingstoke, UK).

Figure 4.3: Algorithm for laboratory handling of vagino-rectal swabs, isolation and identification of GBS.



4.4 RESULTS:

Seven hundred and forty eight women were recruited over two years (2001-3), of which 167 were from the Horton Hospital, Banbury and the remainder from the JRH, Oxford. One hundred and fifty-nine women (21.3%) were carriers of GBS. There was no significant difference between carriage rates at the JRH, a tertiary referral hospital, and at the Horton which is a district general hospital.

4.4.i *Results of demographic and clinical questionnaires:*

For the 167 mothers recruited at the Horton Hospital, further data were available from questionnaires. The demographics of the study population showed that 92.8% of the pregnant women were white-British (Table 4.1) and 91.0% were married (Figure 4.4). First-time pregnancies made up 45% of recruited women (Figure 4.5). There were 170 infants born to 167 women, of which 93 (54.7%) were female.

Table 4.1: GBS carriage study: Ethnic origin of pregnant women at the Horton Hospital.

Ethnic origin	Number (%)
British-white	155 (92.8)
Other-white	7 (4.2)
Asian-other	2 (1.2)
African	1 (0.6)
Irish-white	1 (0.6)
Pakistani	1 (0.6)
<i>Total</i>	<i>167</i>

Figure 4.4: GBS carriage study: Marital status of pregnant women at the Horton Hospital.

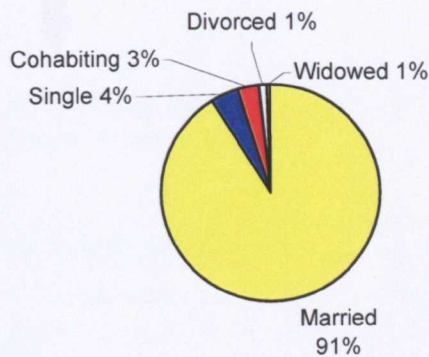
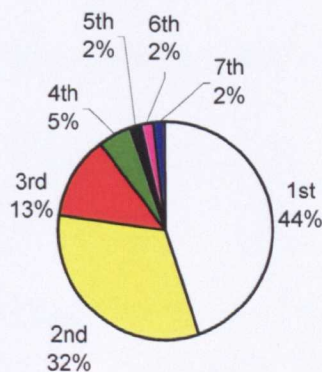


Figure 4.5: GBS carriage study: number of pregnancy at time of study in pregnant women at the Horton Hospital.



Twenty-six women (15.6%) recalled receiving oral antibiotics at some time during their pregnancy (Table 4.2). Approximately 50% of women could not recall why they were treated and what the antibiotic was called. Of those who could remember, urinary sepsis was the most frequent reason and amoxicillin or cephalixin was commonly given. Only 1 of the 26 cases receiving oral antibiotics was carrying GBS vagino-rectally. Eight women had GBS isolated from vaginal swab culture, carried out incidentally during their pregnancy. Only

one of these eight women proved to be carrying GBS at delivery on vagino-rectal sampling. Three of the 8 cases had received oral antibiotics during pregnancy.

Table 4.2 : GBS carriage study: oral antibiotics (PO) given during pregnancy and reason for course of antibiotics.

Reason for antibiotic course		Antibiotics given (PO)	
UTI	9	Amoxicillin	6
ROM	1	Cephalexin	6
Chest infection	2	Erythromycin	2
Do not remember	14	Penicillin	1
		Do not remember	11
<i>Total</i>	<i>26</i>	<i>Total</i>	<i>26</i>

Abbreviations: UTI, urinary tract infection; ROM, rupture of membranes; PO, per oral.

4.4.ii *Results of screening for GBS with vagino-rectal swab culture:*

Twenty-nine mothers (17.4%) from the Horton Hospital were GBS carriers. In 34 (20.4%) mothers a risk factor (fever $>38^{\circ}\text{C}$, prolonged rupture of membranes >18 hours, pre-term delivery <37 weeks) for GBS colonisation was identified¹⁰⁸ (Table 4.3). The correlation between GBS carrier status and identification of a risk factor was not significant (Table 4.3). GBS carrier status was not significantly associated with any risk factor and 24 women with GBS did not develop a risk factor. Twenty-five percent of women would have been treated with IAP in this study using the risk factor approach and a minimum of 21% under the screening approach (CDC guidelines⁶⁶).

Table 4.3: The occurrence of risk factors during pregnancy, with level of significance, in women attending the Horton Hospital.

	GBS carrier (n=29)	GBS negative (n=138)	P- value*	Odds ratios (95% CI)
Instrumental / CS delivery	7	50	0.3	0.6 (0.2-1.5)
Any risk factor	5	29	0.8	0.8 (0.2-2.4)
<i>Fever >38°C</i>	2	9	0.6	1.1 (0-5.8)
<i>PROM >18 hours</i>	2	14	0.5	1.1 (0-5.8)
<i>Preterm <37 weeks</i>	0	3	0.6	0 (0-11)
<i>Fever and PROM</i>	1	3	0.5	1.6 (0.3-3.8)

Abbreviations: CI, 95% confidence intervals; CS, caesarean section; PROM, prolonged rupture of membranes

* Yates-corrected test used, except where values were <5, then one-tailed Fisher exact test used.

Twelve women (7.2%) were given intravenous antibiotics (coamoxiclav, amoxicillin or cefuroxime) during labour. The indication for antibiotic therapy was emergency CS in four cases, two each had fever or prolonged rupture of membranes, one had premature delivery and for 3 cases, the indication was not recorded. The rate of instrumental delivery was 34.1% overall, and was not significantly affected by GBS carrier status.

A single case of early onset GBS neonatal infection occurred in the study period in a premature infant born at 34 weeks to a mother who had not, for logistic reasons been recruited to the study.

4.5 DISCUSSION:

The carriage rate of GBS in pregnant women in the Oxford area is 21.3%. This is a similar rate to that seen in London in 1987 and in Ireland in 1991, but is considerably higher than that seen in North Wales 2002. The methodology used in the North Wales study was similar to that used in this study, vagino-rectal swabs were taken from pregnant women and cultured in selective broth culture. This suggests that geographical variation in GBS carriage rates may be large in the UK. Possible causes for this variation may be socio-economic, due to local patterns of antibiotic use and may be affected by ethnicity of the population in question.

The Horton Hospital, Banbury is a small district general hospital in rural Oxfordshire. This site was chosen as the focus for a questionnaire based epidemiological study of GBS carriage as it is not a tertiary referral unit, in order to avoid a bias towards complicated and high-risk pregnancy in the study. The carriage rate of GBS (17.4%) at the Horton Hospital was not significantly different from that found at the JRH (22.4%).

Demographic questions were included in a questionnaire and were filled in by recruited pregnant women. The demographic profile of the population of women attending the Horton Hospital was not cosmopolitan, the vast majority of women being married and ethnically white-British.

Antibiotic use during pregnancy was fairly common, with 15.6% of women recalling recent oral antibiotic use. The fact that the majority of women could

not recall why or with what drug they had been treated suggests that education of the pregnant woman or the wording of the questionnaire may need to be improved. Of the 8 women who had incidentally had a previous vaginal culture showing GBS, three had received a course of oral antibiotics and only one had a positive culture at delivery. This provides support for the assertion that GBS carriage can be dynamic and that cultures taken within four weeks of delivery are most predictive of GBS carriage status at delivery.

During 2001-3, there were 11 cases of early onset GBS infection admitted to the JRH in a birth cohort of 12032, giving a rate of 0.9/1000 live births. This is in keeping with a previous study of neonatal invasive GBS disease in Oxford⁶⁹.

An important finding was the poor correlation between GBS carriage and the development of risk factors in the studied women. Approximately the same number of women would be treated with antibiotics according to this study under the risk factors based or the screening approach. It is difficult therefore to recommend the risk factor approach over the screening method given that the same numbers of women are likely to be treated with IAP according to both strategies; however, with a lower efficacy of the risk factor approach in the prevention of GBS neonatal disease.

In conclusion, in this UK study of 748 pregnant women, 21.3% were colonised vagino-rectally with GBS. Epidemiological data were collected for a subset of 167 women. Colonisation by GBS was not significantly associated with the previously published risk factors, such as fever, prolonged rupture of membranes

and preterm delivery, although the numbers in each group were small. Twenty-four percent of women would have been given with IAP should a risk-factor strategy be followed and 21% would have received IAP if a screening policy was adopted.

Chapter 5: Development of a Multilocus Sequence Typing System for Group B Streptococcus

5.1 AIMS:

1. To determine suitable genes for use in the GBS MLST system.
2. To design oligonucleotide primers that are reliable in amplification and sequence analysis.
3. To optimise PCR and sequence reactions.

5.2 INTRODUCTION:

Investigation of the epidemiology of infectious diseases is dependant on the ability to characterise strains of a bacterial pathogen. MLST was proposed in 1998¹²⁴ as a general approach to provide accurate and portable data suitable for such study. Recombination is common in populations of many bacteria. Furthermore, areas of the chromosome, where evolution is likely to be rapid and under strong selection pressure will probably be most variable and least suitable for determining the population structure of the species. Housekeeping genes, that is those coding for proteins of essential metabolic functions in the cell, are functionally constrained, not under strong selective pressure and the variation that accumulates in these genes is therefore likely to be neutral. Housekeeping genes, flanked by genes of similar function, are consequently good targets for MLST systems¹²⁵. Most of the MLST systems that have been developed utilise 450 to 500 base-pair fragments of seven genes^{124,134,142,143}. The elements that are critical in the development of a system of MLST and which are the focus of this study, include the choice of genetic loci to be characterised and the design of

oligonucleotide primers for gene amplification and nucleotide sequence determination¹²⁵.

5.3 MATERIALS AND METHODS:

5.3.i *Assembly of collection of isolates:*

A small collection (n=10) of GBS isolates was assembled for the development of the MLST procedure. The isolates were acquired from cases of invasive GBS disease (n=6) and asymptomatic carriage (n=4) from the United Kingdom and Asia.

5.3.ii *Development of Oligonucleotide primers:*

Twelve genes were chosen for study. The genes were those responsible for essential cell metabolism, where variation within the population was presumed to be selectively neutral and the majority had been successfully utilised in MLST of other bacterial pathogens. The NEM316 GBS genome¹⁴⁴ was screened using homologous sequences of selected housekeeping genes from other organisms in order to obtain the GBS sequence of the studied genes. Oligonucleotide primers were then designed from these GBS sequences.

5.3.ii.a *Candidate genes.* The genes studied were the following:- *pgm*, phosphoglucomutase (gbs0904); *purK*, phosphoribosylglycinamide formyltransferase (gbs0003); *pscB*, ribose-phosphate pyrophosphokinase (gbs0017); *cysK*, cysteine synthetase A (gbs0323); *ribF*, riboflavin biosynthesis protein (gbs1032); *adhP*, alcohol dehydrogenase (gbs0054); *pheS*, phenylalanyl tRNA synthetase; *atr*, amino acid transporter (gbs0538); *glnA*, glutamine

synthetase; *sdhA*, serine dehydratase (gbs2105); *glcK*, glucose kinase (gbs0518); *tkl*, transketolase (gbs2105).

5.3.ii.b Oligonucleotide primer design. A nested method was designed utilising 2 sets of primers. The first set of primers were used in the PCR reaction and amplified a large fragment (700 bp) of DNA, the second set of primers were located inside the first set and were used for sequencing, resulting in a sequenced region of about 500 bp of DNA.

The DNA sequences of the study genes were analysed by eye, to detect potential areas with suitable GC content (approaching 50%) for primer design. Runs of 20 base-pairs of DNA within the study genes, to result in an amplicon of suitable size, were entered into the internet based oligonucleotide properties calculator (<http://www.basic.nwu.edu/biotools/oligocalc.html>) for calculation of melting temperature, GC content and the presence of secondary structure associated with reduced efficiency of priming. Primers with favourable qualities were tested against the collection of GBS isolates. If amplification or sequencing were not reliable, then different combinations of primers or new primers were then tested.

From the panel of twelve genes, seven were chosen to make up the GBS MLST system. To determine which genes would be used in the final system, the following factors were considered. Firstly, the diversity of the genes, as measured by the number of different sequences (alleles) identified for that gene, d_H/d_S ratios and the factor π , a measure of nucleotide diversity. Secondly, the genes were grouped together into three groups, group 1 (*adhP*, *pheS*, *atr*), group

2 (*glnA*, *sdhA*, *glcK*, *tkl*) and group 3 (*pgm*, *ribF*, *pseB*, *cysK*). Sequences were concatenated and then trees were generated (using UPGMA) for different combinations of groups (groups 1+2, groups 1+3 and groups 2+3) and analysed for tree morphology, which most closely approximated that when all genes were included.

5.4 RESULTS:

Twelve genes were successfully amplified in PCR reactions and sequenced. In the case of the *purK* gene, only 300 base-pairs could be successfully sequenced. The sequences of the primers are listed in Table 5.1.

Table 5.1: Oligonucleotide nucleotide primers utilised in the MLST system for GBS.

Name and Sequence of Oligonucleotide Primer				
Locus		Forward (5' to 3')	Reverse (5' to 3')	Amplicon size (bp)
<i>adhP</i>	amplification	GTTGGTCATGGTGAAGCACT	ACTGTACCTCCAGCACGAAC	672
	sequencing	GGTGTGTGCCATACTGATTT	ACAGCAGTCACAACCACTCC	498
<i>pheS</i>	amplification	GATTAAGGAGTAGTGGCACG	TTGAGATCGCCCATTGAAAT	723
	sequencing	ATATCAACTCAAGAAAAGCT	TGATGGAATTGATGGCTATG	501
<i>atr</i>	amplification	CGATTCTCTCAGCTTTGTTA	AAGAAATCTCTTGTGCGG AT	627
	sequencing	ATGGTTGAGCCAATTATTTT	CCTTGCTCAACAATAATGCC	501
<i>glnA</i>	amplification	CCGGCTACAGATGAACAATT	CTGATAATTGCCATTCCACG	589
	sequencing	AATAAAGCAATGTTTGATGG	GCATTGTTCCCTTCATTATC	498
<i>sdhA</i>	amplification	AGAGCAAGCTAATAGCCAAC	ATATCAGCAGCAACAAGTGC	646
	sequencing	AACATAGCAGAGCTCATGAT	GGGACTTCACTAAACCTGC	519
<i>glcK</i>	amplification	CTCGGAGGAACGACCATTAA	CTTGTAACAGTATCACCGTT	607
	sequencing	GGTATCTTGACGCTTGAGGG	ATCGCTGCTTTAATGGCAGA	459
<i>tkl</i>	amplification	CCAGGCTTTGATTTAGTTGA	AATAGCTTGTGGCTTGAAA	859
	sequencing	ACACTTCATGGTGATGGTTG	TGACCTAGGTCATGAGCTTT	480

The characteristics of the studied genes are listed in Table 5.3. The gene, *purK*, which was not reliably sequenced for 500bp and showed a much higher diversity was excluded from further analyses. The remaining genes were similar in their amount of diversity.

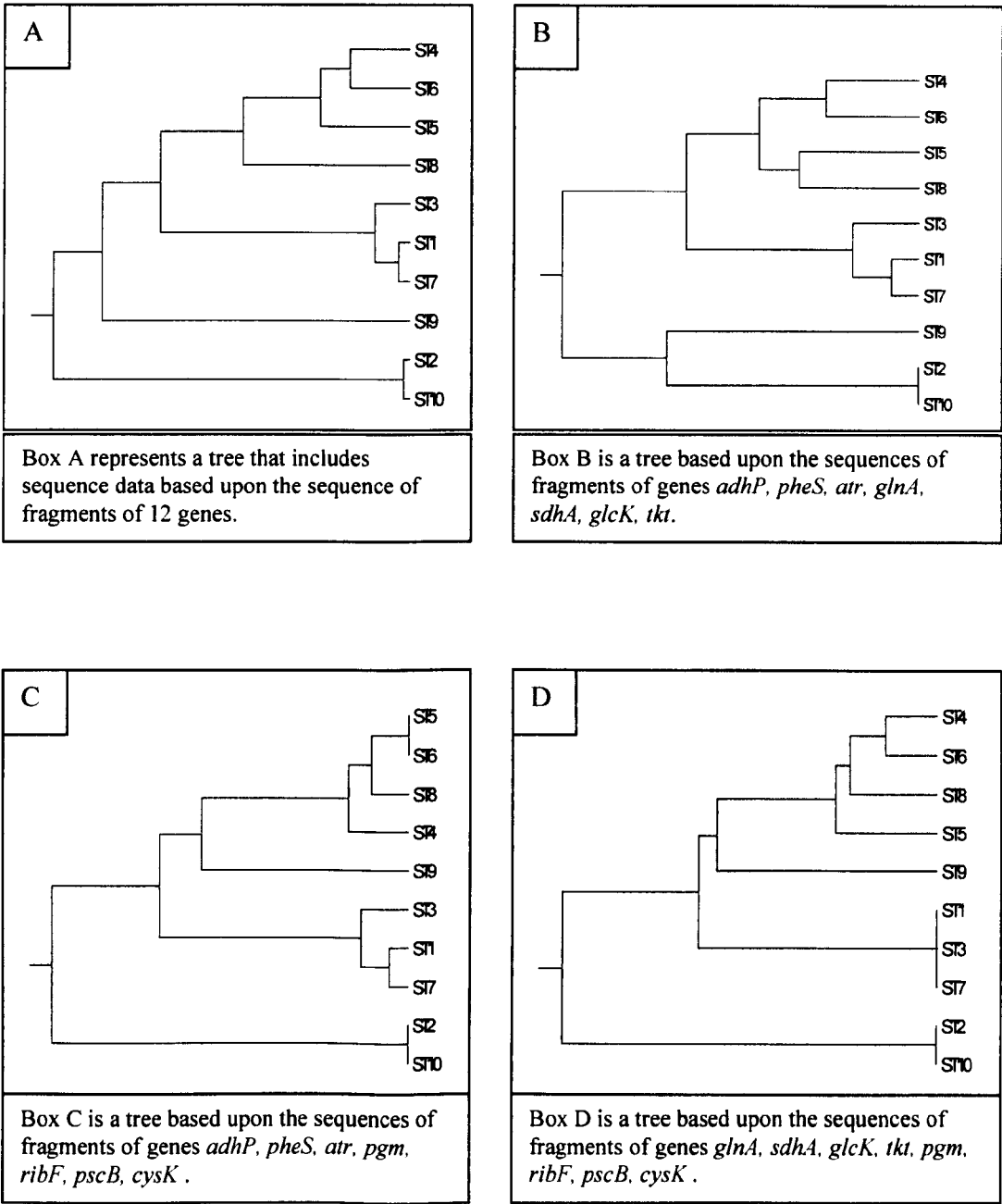
Table 5.3: Measures of diversity in candidate genes utilised in the development of MLST for GBS.

Gene	No. Segregating Sites	Synonymous base changes	Non-Synonymous base changes	No. alleles	%GC	π
<i>adhP</i>	8	5	3	6	0.43	0.0063
<i>pheS</i>	5	4	1	3	0.37	0.0067
<i>atr</i>	9	6	3	6	0.37	0.0076
<i>glnA</i>	4	4	0	3	0.36	0.0054
<i>sdhA</i>	11	8	3	4	0.42	0.012
<i>glcK</i>	5	4	1	3	0.43	0.0073
<i>tkl</i>	5	2	3	3	0.39	0.0069
<i>pgm</i>	3	0	3	2	0.38	0.0066
<i>ribF</i>	3	3	0	2	0.36	0.0071
<i>purK</i>	33	25	8	4	0.51	0.044
<i>pscB</i>	3	0	3	3	0.37	0.004
<i>cysK</i>	4	1	3	2	0.4	0.0078

Trees generated (Figure 5.1) from the 3 groups of genes suggested that the combination of group 1 (*adhP*, *pheS*, *atr*) and group 2 (*glnA*, *sdhA*, *glcK*, *tkl*) resulted in a tree which more closely approximated the tree generated with the sequences of all 11 genes. In addition, it was noted that *ribF*, *cysK*, *pgm* had

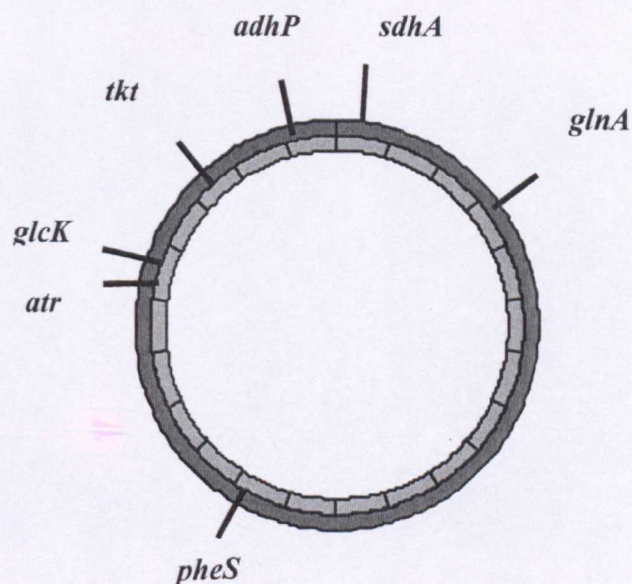
fewer alleles and that *pscB* had the lowest value for π , as support for their exclusion.

Figure 5.1: Trees generated using UPGMA of 10 GBS isolates based upon the concatenated nucleotide sequences of fragments of seven to twelve genes.



The system was then based upon the 7 genes from groups 1 and 2, *adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, *tkl*. The location of these genes around the GBS chromosome is pictured in Figure 5.2, the minimum distance between any two genes was 20 kilobase- pairs.

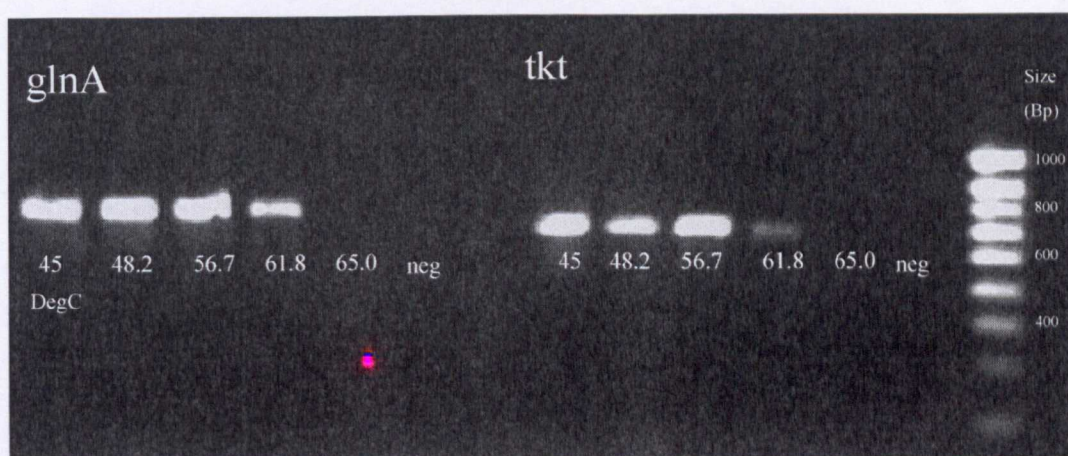
Figure 5.2: Location of genes selected for MLST around the GBS chromosome. The 2,211,485-bp genome is divided into 22 segments (indicated on the inner circle), with each segment representing 100,522 bp.



Temperature gradient PCR was carried out on the seven genes to optimise the annealing temperature that provided a single amplified fragment in good yield.

The annealing temperature was varied between 45 and 65°C. Figure 5.3 demonstrates a photograph of an agarose gel (1%) with the PCR products of two genes, *glnA* and *tkl*, with different annealing temperatures. It can be seen that at 61.8°C, the PCR product band has become fainter, particularly in the case of *tkl*. An annealing temperature of 55°C was selected, ensuring a good amount of PCR product, but high enough to be stringent and low enough to result in successful PCR in most isolates.

Figure 5.3: Photograph of 1% agarose gel labelled with ethidium bromide showing the PCR products of two genes (*glnA* and *tkl*) when PCR was carried out with annealing temperatures from 45 to 65°C.



5.5 DISCUSSION:

The MLST was developed for GBS following the principles established previously¹²⁵. Housekeeping genes, which had been successfully utilised for other organisms were chosen for further study. The system developed was then based upon seven genes, chosen for the favourable characteristics of reliable amplification, adequate diversity and clustering by UPGMA.

The genes utilised for the MLST method were distributed around the GBS chromosome suggesting that they were unlikely to be linked genetically in a single recombinational event.

A nested system was followed, with different oligonucleotide primers being used for PCR and sequencing reactions in order to minimise the likelihood of non-specific sequencing. Gradient PCR reactions were carried out which determined that amplification occurred for all genes at a range of temperatures of up to 60°C. An annealing temperature of 55°C was chosen, as a compromise between successful PCR in a large number of isolates and reducing the likelihood of non-specific binding.

Chapter 6: Validation and assessment of GBS MLST as a typing system.

This chapter has been presented in part as a poster presentation at the XV Lancefield International Symposium of Streptococcal Diseases, Goa, India, 6- 11 October 2002; session P 4.30.

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A: VALIDATION OF THE GBS MLST SYSTEM ON A GLOBAL COLLECTION OF HUMAN ISOLATES.

6.1 AIMS:

To show that the MLST system developed for GBS could be used on a diverse globally-derived collection of isolates from neonates and adults and that the system could distinguish between strains that were different using capsular serotyping and RDP typing.

6.2 INTRODUCTION:

MLST developed for GBS (Chapter 5) was tested against a global collection of human GBS isolates. The collection was intended to represent the full spectrum of human disease and carriage. GBS was compared with two other typing methods, capsular serotyping and restriction digest pattern typing.

6.3 MATERIALS AND METHODS:

6.3.i *Assembly of collection of isolates:*

The study collection consisted of 152 isolates of GBS from North America, New Zealand, Thailand, Singapore, Israel, Japan and the UK. In addition two well-characterised strains were included, the NCTC-8541 strain (isolated from vaginal carriage, Public Health Laboratory, UK) and the NEM316 strain (ATCC12403, isolated from fatal neonatal sepsis, country of origin unknown), whose genome has been fully sequenced¹⁴⁴. The collection was globally diverse and included isolates from asymptomatic carriage as well as those causing human disease. Most capsular serotypes of GBS were represented, although serotypes IV and VII, which are rarely associated with disease in humans, were not included.

6.3.ii *Other methods:*

Methods for capsular serotyping, DNA extraction, PCR, nucleotide sequencing and computational analyses are detailed in Chapter 2, Materials and Methods.

6.4 RESULTS:

6.4.i Collection of GBS isolates:

Table 6.1 further describes the isolate collection.

Table 6.1: GBS isolate collection used for the development of MLST system.

Figures are numbers of isolates (%).

Country	Neonatal		Adult		Totals
	Carried	Invasive	Carried	Invasive	
UK	0	21 (13.9)	20 (13.2)	0	41 (27.1)
USA	0	14 (9.2)	6 (4.0)	2 (1.2)	22 (14.4)
Japan	0	15 (9.9)	30 (19.7)	0	45 (29.6)
New Zealand	2 (1.2)	6 (4.0)	5 (3.3)	10 (6.6)	23 (15.1)
Israel	0	0	3 (2.0)	7 (4.6)	10 (6.6)
Singapore	0	1 (0.6)	0	6 (4.0)	7 (4.6)
Thailand	0	0	3 (2.0)	0	3 (2.0)
Unknown*	0	1 (0.6)	0	0	1 (0.6)
Totals	2 (1.2)	58 (38.2)	67 (44.2)	25 (16.4)	152

* NEM316 (ATCC 12403)

6.4.ii Variation at the seven MLST loci:

The sequences of the seven chosen loci were determined for the 152 isolates and allelic profiles assigned. The alleles defined for the MLST scheme were based on sequence lengths of between 459 (*glcK*) to 519 base pairs (*sdhA*). Between 4 (*glcK*) and 11 alleles (*adhP*) were present at each locus. The average number of alleles at each locus was 6.4, providing the potential to distinguish 4.4×10^5 different genotypes. The proportion of variable nucleotide sites present in the chosen housekeeping genes ranged from 1.2% (*glnA*) to 2.5% (*sdhA*) (Table 6.2 and Figure 6.1). The proportions of nucleotide alterations that changed the amino acid sequence (non-synonymous substitutions, d_n) and the proportions of silent changes (synonymous substitutions, d_s) were calculated for each gene.

Using this data the d_n/d_s ratios were calculated for all seven loci and were all <1 (Table 6.2).

Table 6.2: Characteristics of loci included in the GBS MLST system.

Locus	Size of sequenced fragment (bp)	No. of alleles identified	No. of polymorphic nucleotide sites (%)	%GC	d_n/d_s	Position in GBS genome (bp) ¹⁴⁴
<i>adhP</i>	498	11	12 (2.4)	43.1	0.13	72,286
<i>pheS</i>	501	5	7 (1.4)	37.1	0.17	912,817
<i>atr</i>	501	8	12 (2.4)	36.9	0.14	560,085
<i>glnA</i>	498	6	6 (1.2)	35.7	0.12	1,868,862
<i>sdhA</i>	519	6	13 (2.5)	41.4	0.12	2,179,923
<i>glcK</i>	459	4	7 (1.5)	42.6	0.13	538,770
<i>tkt</i>	480	5	8 (1.7)	38.9	0.42	287,111

Abbreviations: *adhP*, alcohol dehydrogenase (gbs0054); *pheS*, phenylalanyl tRNA synthetase; *atr*, amino acid transporter (gbs0538); *glnA*, glutamine synthetase; *sdhA*, serine dehydratase (gbs2105); *glcK*, glucose kinase (gbs0518); *tkt*, transketolase (gbs2105)

Figure 6.1: Polymorphic nucleotide sites in GBS MLST genes.

<p><i>adhP</i></p> <p>1122222233 261511234589 231013908546</p> <p>ALLELE1 GGGGAATGCGCAA ALLELE2 ...G.C...T.. ALLELE3 T..G.C...T.. ALLELE4 ..AG...T.T.. ALLELE5 ...GGC...T.G ALLELE8 ..AG..A..T.. ALLELE9 ..AG.....T.. ALLELE10 ..AG.....TG. ALLELE11 ..AG.....AT.. ALLELE12 .A..... ALLELE13 ...G.....</p>	<p><i>pheS</i></p> <p>223334 1393499 3146131</p> <p>ALLELE1 CCCGAAA ALLELE2G ALLELE3 .TTC... ALLELE4 .TT.GC. ALLELE9 T.....</p>
<p><i>atr</i></p> <p>11122333334 335767045679 323276001683</p> <p>ALLELE1 GGGCAGCGAAGG ALLELE2 A...G.TAT.A. ALLELE3 A...GTTAT.A. ALLELE4T.A. ALLELE5 .AA.....T... ALLELE6TGA. ALLELE7T.AA ALLELE8 A..TGTTAT.A.</p>	<p><i>glnA</i></p> <p>13344 363824 656795</p> <p>ALLELE1 CGGATT ALLELE2C. ALLELE3 T..GCC ALLELE4CC ALLELE5 .T..C. ALLELE8 ..A.C.</p>
<p><i>sdhA</i></p> <p>1122233 5558891202836 0696900011468</p> <p>ALLELE1 CGTGCCTGGATAT ALLELE2 TACA.....GC ALLELE3 .A.A.TC..GCGC ALLELE5 .A.A.TCA.GCGC ALLELE6A..... ALLELE8 TACA....A..GC</p>	<p><i>glcK</i></p> <p>112444 9188133 6202025</p> <p>ALLELE1 TGGTCCT ALLELE2 ..AC..C ALLELE3 G.ACT.. ALLELE4 .TAC.T.</p>
	<p><i>tkt</i></p> <p>123334 69742566 99905920</p> <p>ALLELE1 GGCCGCCG ALLELE2 AA....AA ALLELE3T.. ALLELE4 AA..... ALLELE6 .AATA.A.</p>

6.4.iii

Relatedness of GBS isolates:

The 152 isolates were resolved into 29 STs, 14 of which were identified only once (Table 6.3, Table 6.4). One hundred and one isolates (66.5% of the data set) were represented by one of four STs, ST-1, ST-17, ST-19 and ST-23. The most common ST (ST-17) was identified 44 times in the dataset, followed by ST-1 (21 isolates), ST-19 (20 isolates) and ST-23 (16 isolates). ST-3 was not included in this dataset, but had been identified in a pilot study.

Table 6.3: The collection of 152 isolates of GBS described according to ST, country of isolation, host type, epidemiology and capsule.

Isolate No.	ST	Country	Host	Epidemiology	Capsule
IS11	1	Is	A	C	V
IS19	1	Is	A	I	V
IS2	1	Is	A	I	V
U64	1	J	A	C	III
U65	1	J	A	C	III
U88	1	J	A	C	VI
U89	1	J	A	C	VI
U90	1	J	A	C	VI
U92	1	J	A	C	VI
U93	1	J	A	C	VIII
U94	1	J	A	C	VIII
U95	1	J	A	C	VIII
U96	1	J	A	C	VIII
NZ14	1	NZ	N	I	V
NZ18	1	NZ	A	C	V
NZ23	1	NZ	A	I	V
T3	1	T	A	C	NT
Z12	1	UK	A	C	V
Z84	1	UK	A	C	V
Z95	1	UK	A	C	V
UK22	1	UK	N	I	IB
IS28	2	Is	A	I	NT
NZ7	2	NZ	A	I	II
U63	4	J	A	C	IA
UK6	4	UK	N	I	IA
U62	5	J	A	C	IA
Z78	6	UK	A	C	IB
U72	7	J	A	C	IA
U75	7	J	N	I	IA
U71	7	J	N	I	IA
U79	8	J	A	C	IB
NZ19	8	NZ	A	I	IB
NZ4	8	NZ	A	I	IB
A8	8	S	A	I	NT
Z111	8	UK	A	C	IB
Z72	8	UK	A	C	IB
UK13	8	UK	N	I	IB
IS13	9	Is	A	C	IB
U78	10	J	N	I	IB
NZ17	10	NZ	A	I	IB
NZ20	10	NZ	A	I	II
Z73	10	UK	A	C	IB
Z41	10	UK	A	C	NT
A2	11	S	A	I	III
A3	11	S	A	I	III
A4	11	S	A	I	III
A5	11	S	A	I	III
A6	11	S	A	I	III
U80	12	J	N	I	IB
U81	12	J	N	I	IB
Z69	12	UK	A	C	IB
T5	13	T	A	C	VI
T1	14	T	A	C	VI
NZ15	15	NZ	A	I	IB
NZ16	15	NZ	N	I	IB
IS56	16	Is	A	I	IA
U11	17	J	A	C	III
U23	17	J	A	C	III
U25	17	J	A	C	III
U3	17	J	A	C	III
U4	17	J	A	C	III
U5	17	J	A	C	III
U1	17	J	N	I	III
U12	17	J	N	I	III
U13	17	J	N	I	III
U14	17	J	N	I	III
U15	17	J	N	I	III
U2	17	J	N	I	III
U26	17	J	N	I	III
U24	17	J	N	I	III
NZ10	17	NZ	N	I	III
Z34	17	UK	A	C	III
Z37	17	UK	A	C	III
UK3	17	UK	N	I	III
UK4	17	UK	N	I	III
UK5	17	UK	N	I	III

Isolate No.	ST	Country	Host	Epidemiology	Capsule
UK8	17	UK	N	I	III
UK10	17	UK	N	I	III
UK12	17	UK	N	I	III
UK15	17	UK	N	I	III
UK17	17	UK	N	I	III
UK20	17	UK	N	I	III
UK21	17	UK	N	I	III
UK18	17	UK	N	I	III
U21	17	USA	A	I	III
U22	17	USA	A	I	III
U29	17	USA	A	C	III
U10	17	USA	N	I	III
U17	17	USA	N	I	III
U19	17	USA	N	I	III
U20	17	USA	N	I	III
U27	17	USA	N	I	III
U28	17	USA	N	I	III
U31	17	USA	N	I	III
U7	17	USA	N	I	III
U8	17	USA	N	I	III
U9	17	USA	N	I	III
U18	17	USA	N	I	III
U30	17	USA	N	I	III
U32	17	USA	N	I	III
UK11	18	UK	N	I	III
U54	19	J	A	C	III
U84	19	J	A	C	V
NZ1	19	NZ	A	I	III
NZ11	19	NZ	A	C	III
NZ2	19	NZ	N	I	III
NZ21	19	NZ	A	I	III
NZ3	19	NZ	N	C	III
UK16	19	UK	A	C	III
Z101	19	UK	A	C	III
Z117	19	UK	A	C	III
Z77	19	UK	A	C	II
8541	19	UK	A	C	NT
Z50	19	UK	A	C	III
UK7	19	UK	N	I	III
UK19	19	UK	N	I	III
U55	19	USA	A	C	III
U56	19	USA	A	C	III
U57	19	USA	A	C	III
U58	19	USA	A	C	III
U59	19	USA	A	C	III
UK1	20	UK	N	I	III
U53	21	J	N	I	III
IS1	22	Is	A	I	II
IS12	22	Is	A	C	II
IS9	23	Is	A	I	III
U69	23	J	A	C	IA
U70	23	J	A	C	IA
U60	23	J	A	C	III
NZ12	23	NZ	N	I	IA
NZ13	23	NZ	N	C	IA
NZ22	23	NZ	A	I	IA
NZ5	23	NZ	N	I	III
NZ6	23	NZ	A	C	IA
NZ8	23	NZ	A	C	IA
NZ9	23	NZ	A	I	IA
A7	23	S	N	I	IA
Z81	23	UK	A	C	IA
Z87	23	UK	A	C	V
UK14	23	UK	N	I	IA
NEM316	23	NK	N	I	III
Z18	24	UK	A	C	IA
U61	25	USA	N	I	III
U86	26	J	A	C	V
U87	26	J	A	C	V
UK2	26	UK	N	I	V
IS31	27	Is	A	I	III
U82	28	J	A	C	II
U83	28	J	A	C	II
UK9	28	UK	N	I	II
U16	29	J	N	I	III
U85	30	J	A	C	V

Abbreviations: ST, sequence type; NT, non-typeable; NK, not known; A, adult; N, neonatal; I, invasive isolate; C, carried isolate; J, Japan; UK, United Kingdom; Is, Israel; NZ, New Zealand; T, Thailand; S, Singapore.

Table 6.4: Characteristics of GBS isolates according to ST.

ST	Allelic profile*	No.	Serotype (No.)	Source (No.)	Country of origin (No.)
1	1,1,2,1,1,2,2	21	V(9), VIII(4), VI(4), III(2), IB(1), NT(1)	AC (16), AI (3), NI (2)	J(10), UK (4), Is(3), NZ (3), T(1)
2	1,1,3,1,1,2,2	2	II (1), NT (1)	AI(2)	Is (1), NZ (1)
4	1,1,4,1,1,3,4	2	IA (2)	AC (1), NI (1)	J (1), UK (1)
5	12,1,4,1,1,3,4	1	IA (1)	AC (1)	J (1)
6	9,1,2,1,3,2,2	1	IB (1)	AC (1)	UK (1)
7	10,1,2,1,3,2,2	3	IA (3)	AC (1), NI (2)	J (3)
8	4,1,4,1,3,3,2	7	IB (6), NT(1)	AC(4), NI(1), AI(2)	UK(3), NZ(2), J(1), S(1)
9	8,1,4,1,3,3,2	1	IB (1)	AC(1)	Is(1)
10	9,1,4,1,3,3,2	5	IB(3), II(1), NT(1)	AC(2), NI(1), AI(2)	UK(2), NZ(2), J(1)
11	9,3,7,1,3,3,2	5	III(5)	AI(5)	S(5)
12	10,1,4,1,3,3,2	3	IB (3)	NI(2), AC(1)	J(2), UK(1)
13	11,3,4,1,3,3,2	1	VI(1)	AC(1)	T(1)
14	1,1,2,1,5,2,2	1	VI(1)	AC(1)	T(1)
15	9,1,4,1,5,3,2	2	IB(2)	AI(1), NI(1)	NZ(2)
16	1,1,4,1,6,3,4	1	IA(1)	AI(1)	Is(1)
17	2,1,1,2,1,1,1	44	III(44)	NI(33), AC(9), AI(2)	US(16), J(14), UK(13), NZ(1)
18	3,1,1,2,1,1,1	1	III(1)	NI(1)	UK(1)
19	1,1,3,2,2,2,2	20	III(17), II(1), V(1), NT(1)	AC(14), NI(3), AI(2), NC(1)	UK(8), NZ(5), US(5), J(2)
20	1,2,3,2,2,2,2	1	III(1)	NI(1)	UK(1)
21	1,9,3,2,2,2,2	1	III(1)	NI(1)	J(1)
22	13,3,1,3,1,1,1	2	II(2)	AC(1), AI(1)	Is(2)
23	5,4,6,3,2,1,3	16	IA(11), III(4), V(1)	AC(7), NI(5), AI(3), NC(1)	NZ(7), UK(3), J(3), Is(1), S(1), NK(1)
24	5,4,4,3,2,3,3	1	IA(1)	AC(1)	UK(1)
25	5,4,6,3,8,1,3	1	III(1)	NI(1)	US(1)
26	1,1,5,4,1,4,6	3	V(3)	AC(2), NI(1)	J(2), UK(1)
27	1,1,3,4,2,2,2	1	III(1)	AI(1)	Is(1)
28	1,1,3,5,2,2,2	3	II(3)	AC(2), NI(1)	J(2), UK(1)
29	2,1,1,8,1,1,1	1	III(1)	NI(1)	J(1)
30	1,1,8,2,2,2,2	1	V(1)	AC(1)	J(1)

Abbreviations: see Table 6.3.

* Allelic profiles for each gene are presented in the order, *adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, *tki*.

UPGMA was used to construct a dendrogram from the matrix of pairwise differences between the concatenated sequences of 29 STs of all 152 isolates (Figure 6.2). BURST grouped the isolates into 7 lineages (Table 6.5), which approximated well with the clusters of STs obtained by UPGMA. Split decomposition, using concatenated sequences was also used to analyse the relationships between STs (Figure 6.3). A naming system for complexes of STs was adopted. Each complex was named after the central ST identified by BURST. If only 2 STs were identified in a complex, then that complex was named after the ST, which was most prevalent. In general, the three methods clustered the STs into the same seven clusters. There were some differences noted. In particular, ST-14 was located within ST-6 complex by UPGMA and split decomposition, but BURST placed this ST within ST-1 complex. ST-11 and ST-13 are within the ST-10 complex by the methods of UPGMA and split decomposition but was not allocated to a BURST group. Similarly, ST-24 was not included within ST-23 complex by BURST. ST-1 complex, is very closely related to ST-19 and its identity as a separate split is not entirely clear from the trees drawn.

Table 6.5: Analysis of clustering of STs according to BURST (Singletons: ST-11, ST-13, ST-22, ST-24, ST-26).

BURST group	ST-complex	Central ST	STs
1	1	1	1, 2,14
2	4	4	4, 5,16
3	6		6,7
4	10	10	8, 9,10,12,15
5	17	17	17,18,29
6	19	19	19,20,21,27,28,30
7	23		23,25

Figure 6.2: UPGMA dendrogram generated from concatenated sequences showing genetic relationships between 29 STs and naming of clonal complexes.

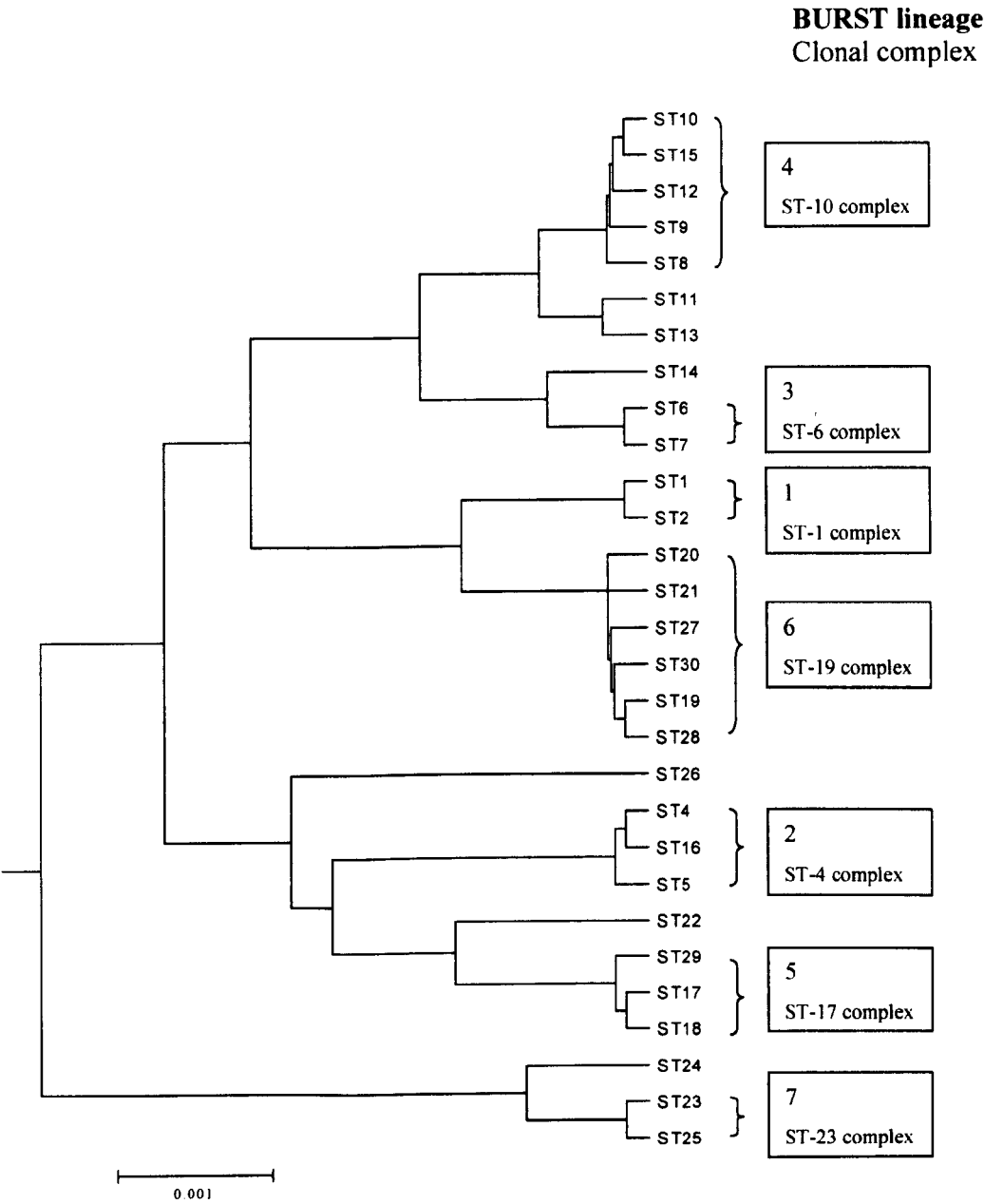
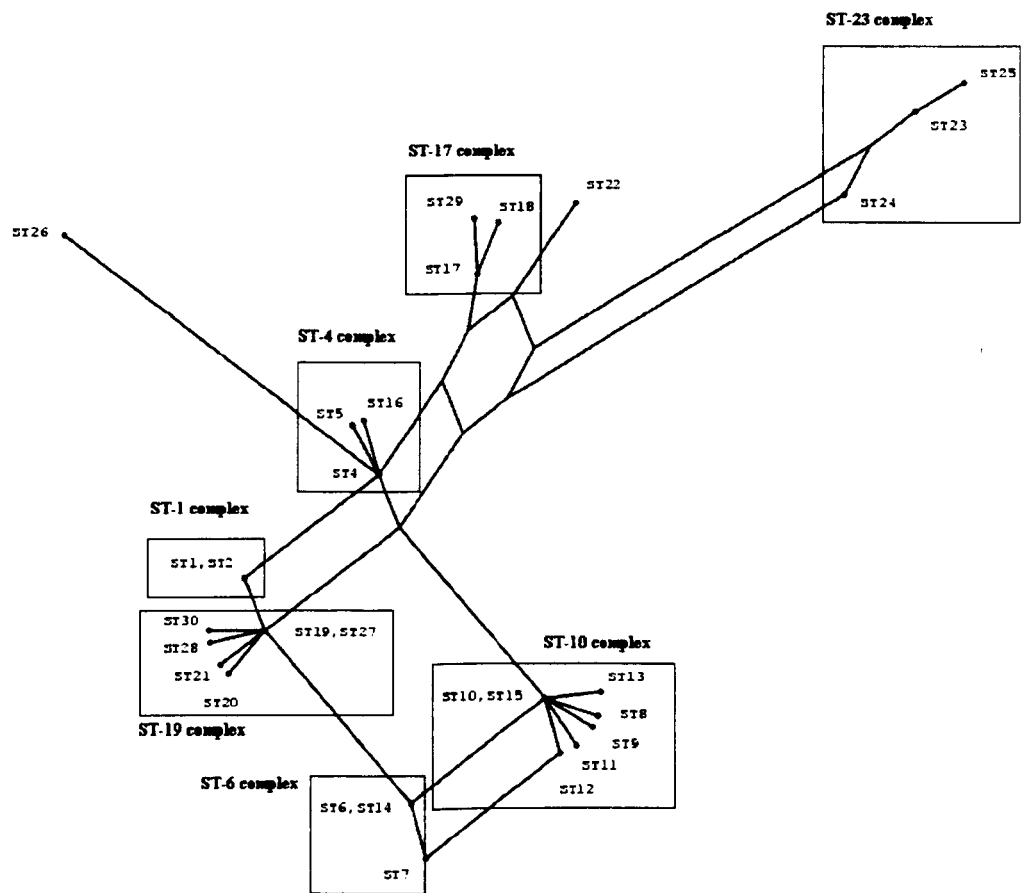


Figure 6.3: Tree drawn by split decomposition analysis of 29 STs with clonal complexes indicated by boxes.



6.4.iv ***Relationship between ST, capsular serotype and Restriction***

Digest Patterns (RDP):

Capsular serotype was known for all 152 isolates (Table 6.3 and Table 6.4) and there was complete correlation between capsular serotyping results between three laboratories. Five isolates proved to be non-typeable. Serotype III was most common (78 isolates, 44 of which belonged to ST-17), followed by, serotype IA (19 isolates), IB (17 isolates), V (15 isolates), II (8 isolates), VI (6 isolates) and VIII (4 isolates). Serotypes IV and VII were not represented in the dataset. Capsular serotype was generally not restricted to specific STs and four STs contained isolates with different capsular serotypes.

RDP typing results were known for 40 of the isolates within the dataset (Table 6.6). RDP type correlated closely with ST, isolates of the same RDP type were identical by MLST or only differed at a single locus.

Table 6.6: Restriction Digest Pattern (RDP) compared with Sequence Type for 40 GBS isolates.

ST	Allelic profile	RDP type	Number of isolates
17	2,1,1,2,1,1,1	III-3	30
29	2,1,1,8,1,1,1	III-3	1
19	1,1,3,2,2,2,2	III-2	6
21	1,9,3,2,2,2,2	III-2	1
23	5,4,6,3,2,1,3	III-1	1
25	5,4,6,3,8,1,3	III-1	1

6.4.v *Relationship between lineage, host and country of origin:*

The dataset presented in this study was not specifically designed to investigate the relationship between isolates and country or host of origin. However, the following observations can be made (Table 6.3). ST-1 and ST-19 were significantly associated with carriage (Yates corrected, $p=0.004$ and $p=0.008$, respectively) and several different capsular serotypes were represented in these STs. The 44 isolates within ST-17 were all serotype III. ST-17 was significantly associated with invasive neonatal disease (Yates corrected, $p=0.0000001$). ST-23 contains serotype IA isolates (11/16, 68.6%) from carriage and invasive disease. ST-1, ST-19, ST-17 and ST-23 each contain isolates isolated from Australasia, Europe, Asia and North America, suggesting global dispersal.

6.5 DISCUSSION:

A multilocus typing scheme for GBS based on seven housekeeping genes was validated using a world-wide collection of capsular-typed isolates that included a sub-group previously characterized by RDP.

The percentage of variable sites (1.2 to 2.5%) in the seven selected GBS genes was comparable to that seen by Tettellin et al¹⁴⁵ in their analysis of sequence variation in 19 genes from 11 GBS strains. The percentage of variable sites is less than that seen in the related species, group A streptococcus¹⁴³ (5.1 to 7.6%) and considerably less than *Campylobacter jejuni*¹⁴² (9.2 to 21.7%), a Gram-negative organism. The d_N/d_S ratios for the seven GBS genes were all less than one, which suggests that there is selection against amino acid change and is consistent with most of the variation being selectively neutral. The genes chosen were distributed around the chromosome and were located in the same approximate location in both of the published GBS genome sequences, NEM316¹⁴⁴ and 2603 V/R¹⁴⁵. MLST results for the two published GBS genome sequences showed that NEM316 was ST-23 and that 2603 V/R was a single locus variant of ST-19.

A website for GBS MLST was set up and can be accessed at the following address, <http://sagalactiae.mlst.net>.

The aim of this work was to establish an MLST typing system, however there were sufficient numbers of isolates to make early observations about the population structure of GBS. The most common STs in the dataset were ST-1,

ST-17, ST-19 and ST-23. These four STs represented two-thirds of the isolate collection. ST-19 and ST-1 contain several different capsular serotypes, and were significantly associated with the carriage state. ST-17 is more homogeneous and consists of serotype III isolates predominantly associated with neonatal invasive disease. The STs were grouped together in similar fashion by UPGMA, SplitsTrees and BURST.

The MLST findings are in accord with the results of Musser et al¹³. These authors used MLEE to study the population structure of GBS. They found that 2 distantly related evolutionary lineages of GBS could be distinguished. The first lineage contained a single electrophoretic type (ET-1) and consisted of serotype III isolates, which had been isolated from neonatal disease. This presumably corresponds to the ST-17 of MLST. The second lineage of MLEE was more diverse and contained several subdivisions and numerous ETs, which may correspond to the ST-19 complex or ST-1 complex, which are more diverse, with several STs and different capsular serotypes. Similar relationships between GBS isolates have also been found using RDP typing¹⁴⁶ and PFGE¹¹.

MLST shows that isolates with the same ST can have different capsular serotypes. This could imply that the MLST scheme has insufficient discriminatory power and groups isolates that are not closely related in genotype. However, a similar variation in the serotype of isolates within a single genotype was also shown by MLEE¹³. The variation in serotype within a single ST, and the presence of genetically diverse isolates with the same serotype, suggest that the capsular biosynthetic genes of GBS are subject to relatively frequent

horizontal gene transfer, as is seen in *Streptococcus pneumoniae*¹⁴⁷. It has been demonstrated that a single gene confers serotype specificity in GBS of capsular types III and IA¹⁴⁸ and recombinational replacement of this gene with that from an isolate of a different serotype would result in a change of capsular type.

However, thus far, horizontal transfer of capsular genes has not been shown for GBS other than in the laboratory. An alternative, perhaps less likely explanation is, that capsular serotyping may be prone to mistakes and is difficult to interpret.

In conclusion, the GBS MLST system appears to be sufficiently discriminatory for epidemiological studies and provides a precise and unambiguous way of characterising isolates of GBS isolates. A website providing information about allele sequences and sequence types has been set up. Results have confirmed previous findings that a single clone of GBS (ST-17) seems to be frequently represented in neonatal invasive disease.

B: EVALUATION OF GBS MLST AS A TYPING SYSTEM.

6.6 AIMS:

To determine the efficiency of GBS MLST as a typing method in comparison to the current gold standard method, capsular serotyping.

6.7 INTRODUCTION:

In the evaluation of a new typing system, it is necessary to consider the typability, reproducibility and discrimination of the new technique, and compare these to the “gold standard” typing mechanism. In the case of GBS, the developed MLST system is compared with capsular serotyping.

6.8 MATERIALS AND METHODS:

MLST was compared with capsular serotyping using a collection of 299 GBS isolates collected from the Oxford area as a result of enhanced surveillance for invasive neonatal infection and a carriage study of asymptomatic carriage of GBS in pregnant women, as previously described (Chapters 3,4). Methodology for MLST and for capsular serotyping is described in Materials and Methods (Chapter 2).

Typability (%) was calculated from the number of isolates that could be allocated to a sequence type for MLST and for capsular serotyping.

Reproducibility (%) was assessed from the proportion of MLST (n=60) reactions, which were repeated at random and yielded the same result. Reactions were repeated from the PCR stage onwards. Measures of discrimination (D) and 95%

CI were calculated according to equations described above (Chapter 1, Introduction).

6.9 RESULTS:

MLST and capsular serotyping by latex agglutination were carried out on 299 isolates. The results are shown in Tables 6.7.

Table 6.7: Sequence types defined by MLST and Capsular Serotypes, according to frequency of identification in 299 strains of GBS.

ST	Number	Capsular Serotype	Number
17	52	III	113
1	50	IA	61
23	50	V	55
19	40	IB	35
8	17	II	22
12	11	VI	4
28	7	VII	2
2	6	IV	1
10	6	VIII	1
22	5	Non- Typeable	5
24	5		
6	4		
7	3		
4	2		
26	2		
107	2		
109	2		
115	2		
130	2		
Other	31		
Total	268	Total	299

Footnote to Table 6.7: Other STs identified only once in collection: ST-9, 18, 20, 27, 65, 78, 88, 103, 104, 106, 108, 110, 111, 112, 113, 114, 116, 117, 118, 128, 129, 131, 132, 133, 134, 136, 137, 138, 139, 140, 141.

6.9.i Typability:

All 299 (100%) were successfully typed by MLST. Five of 299 (1.7%) isolates were non-typeable by capsular serotyping.

6.9.ii Reproducibility:

Sixty of 2093 (2.9%) MLST reactions were repeated, with all reactions identifying the same allele when repeated, giving a repeatability of 100%. Reproducibility was not carried out for latex agglutination results. A subset of isolates (n=39) had capsular serotyping carried out in a second laboratory, using capillary agglutination rather than latex agglutination. The results of capsular serotyping were concordant between the two methods for all 39 isolates.

6.9.iii Discrimination:

Simpson's index of diversity (D) and 95% CI were calculated for the capsular serotyping by latex and for MLST on the dataset. The results are shown in Table 6.8. It can be seen that D was higher for MLST compared to capsular serotyping. The 95% CI do not overlap, suggesting that this difference is significant.

Table 6.8: Index of Diversity (D) and 95% CI for MLST and capsular serotyping for 299 isolates of GBS.

	D	95% CI
Capsular serotyping	0.77	0.74 – 0.79
MLST	0.89	0.88 – 0.91

6.10 DISCUSSION:

MLST has been carried out on 299 GBS isolates and compared with the gold standard method of typing GBS, capsular serotyping.

MLST performs well on all three factors assessed, typability, reproducibility and discrimination. In particular, all isolates could be allocated an ST, whereas 1.7% were non-typeable by capsular serotyping. On repetition of a proportion of MLST reactions, there was high reproducibility with no misidentification of alleles or STs. Additionally, MLST proved to be more discriminating than capsular serotyping (D value, 0.89 compared to 0.77).

In conclusion, MLST is a discriminatory and reproducible typing method for GBS.

Chapter 7: The Population Biology of GBS in carried and invasive isolates from Oxford, UK

7.1 AIMS:

To study the population biology of carried and invasive neonatal GBS from the Oxford area. To estimate the invasive potential of GBS STs in the causation of neonatal disease.

7.2 INTRODUCTION:

A collection of carried GBS from pregnant women were collected over a 2-year period from women attending antenatal care in the Oxford area. A collection of GBS, representing cases of both early and late onset neonatal disease from the Oxford area were assembled both retrospectively from stored isolates and prospectively from enhanced surveillance. These two collections of GBS isolates were put together to represent the ecology of GBS in neonatal infection and formed the basis of a more detailed investigation of the population structure of GBS in this setting.

7.3 MATERIALS AND METHODS:

Methods for assembly of isolate collections have been described in Chapters 3 and 4. Methods for MLST and computational analyses have been described in Chapter 2. Specifically, concatenated sequences representing the sequence types seen were entered into the MEGA and SplitsTrees programmes. Tests of nucleotide diversity (π) and pairwise differences (Π) were calculated using MEGA. Simpson's Index of diversity (D) was calculated for the carried and invasive population. Ninety-five percent CI around the index were calculated

according to described methods (Chapter 2). Invasiveness of STs was estimated by calculation of odds ratios from 2x2 tables.

7.4 RESULTS:

7.4.i *Characteristics of the study population:*

The study population of GBS consisted of 299 isolates, of which 109 were from invasive neonatal disease and 190 were from asymptomatic carriage in pregnant women. All isolates were collected from the Oxford area. MLST was performed on the entire collection and showed that there were 51 STs identified, of which, carried isolates consisted of 38 STs (Table 7.1) and invasive isolates, 29 STs (Table 7.2). The most prevalent STs in the carried population were, ST-23, ST-19 and ST-1 accounting for some 51.6%. In contrast invasive isolates were allocated to ST-17 in 30.3% of cases, with ST-1 (15.6%), ST-23 (11.9%) and ST-19 (11.0%) occurring less frequently.

7.4.ii *Capsular serotyping:*

Examination of capsular serotype revealed that, in the case of the more common STs, only ST-17 was homogeneous, with all 52 isolates of ST-17 being capsular serotype III. ST-23 was predominantly associated with capsular serotype IA (47/50 isolates, 94.0%), ST-1 with serotype V (39/50, 78.0%), ST-8 with serotype IB (15/17, 88%) and ST-19 with serotype III (37/40, 92.5%).

Table 7.1: Carried GBS isolates from Oxford with ST, allelic profile and capsular serotype (in order of frequency of identification).

ST	Number (%)	Capsular Serotype (Number)
23	37 (19.5)	IA (35), V (2)
1	33 (17.4)	V(25), VI(2), VII(2), VIII(1), IV(1), II(1), NT(1)
19	28 (14.7)	III (25), II (3)
17	19 (10.0)	III (19)
8	12 (6.3)	IB (12)
12	10 (5.3)	IB (6), II (2), V (1), NT (1)
10	5 (2.6)	II (3), IB (2)
22	5 (2.6)	V (4), II (1)
28	5 (2.6)	II (3), III (1), NT (1)
24	4 (2.1)	IA (4)
2	3 (1.6)	V (1), VI (1), II (1)
6	3 (1.6)	IB (2), VI (1)
4	1 (0.5)	IA
7	1 (0.5)	IA
26	1 (0.5)	V
65	1 (0.5)	IB
78	1 (0.5)	II
88	1 (0.5)	IA
103	1 (0.5)	IA
104	1 (0.5)	IB
107	1 (0.5)	III
110	1 (0.5)	V
111	1 (0.5)	III
112	1 (0.5)	III
113	1 (0.5)	IB
114	1 (0.5)	IA
115	1 (0.5)	III
116	1 (0.5)	IB
117	1 (0.5)	V
118	1 (0.5)	IB
128	1 (0.5)	III
129	1 (0.5)	III
130	1 (0.5)	V
131	1 (0.5)	III
132	1 (0.5)	IA
139	1 (0.5)	V
140	1 (0.5)	II
141	1 (0.5)	V

Table 7.2: Invasive GBS isolates from Oxford with ST, allelic profile and capsular serotype (in order of frequency of identification).

ST	Number (%)	Capsular Serotype (Number)
17	33 (30.3)	III (33)
1	17 (15.6)	V (14), III (2), IB (1)
23	13 (11.9)	IA (12), III (1)
19	12 (11.0)	III (12)
8	5 (4.6)	IB (3), IA (1), III (1)
2	3 (2.8)	NT (2), II (1)
7	2 (1.8)	IA (1), V (1)
28	2 (1.8)	II (2)
109	2 (1.8)	III (2)
4	1 (0.9)	IA
6	1 (0.9)	IB
9	1 (0.9)	IB
10	1 (0.9)	IB
12	1 (0.9)	IB
18	1 (0.9)	III
20	1 (0.9)	III
24	1 (0.9)	IA
26	1 (0.9)	V
27	1 (0.9)	III
106	1 (0.9)	III
107	1 (0.9)	III
108	1 (0.9)	III
115	1 (0.9)	III
130	1 (0.9)	II
133	1 (0.9)	III
134	1 (0.9)	II
136	1 (0.9)	II
137	1 (0.9)	III
138	1 (0.9)	III

7.4.iii *Representation of population by phylogenetic tree:*

7.4.iii.a *Distance versus discrete tree-building methods.* Two

methods of tree building were used to analyse the data. Firstly the maximum parsimony method was used, this method utilises the data of individual

nucleotide sites. Figure 7.1 shows the maximum parsimony tree for the data. The data are grouped into the same groups or complexes defined by BURST (Chapter 6), which have been called ST-1 complex, ST-6 complex, ST-10 complex, ST-17 complex, ST-19 complex and ST-23 complex. The statistical support for the resultant tree was tested and bootstrap values have been added to the branches. The bootstrap values associated with the branches of the maximum parsimony tree are all less than 70%, which suggests that the tree does not reliably represent the relationships contained within the dataset.

The neighbour joining tree, which is generated using a different algorithm to maximum parsimony and is based upon measurement of evolutionary distance between pairs of sequences, was generated. Although the exact relationship of the STs within each complex is different, the same clustering of STs into complexes is seen. The ordering of the complexes is different however (Figure 7.2).

7.4.iii.b Tree-building with removal of long-branches. Given the finding that there is conflict within the tree suggested by low bootstrap values, the long branches of more distantly related strains, shown in the trees (ST-4, 26,103,141) have been removed from the dataset and a UPGMA tree resulting from this dataset is shown in Figure 7.3. This analysis shows that the complexes around ST-1, ST-6, ST-17, ST-19 and ST-23 are associated with bootstrap values of greater than 70%.

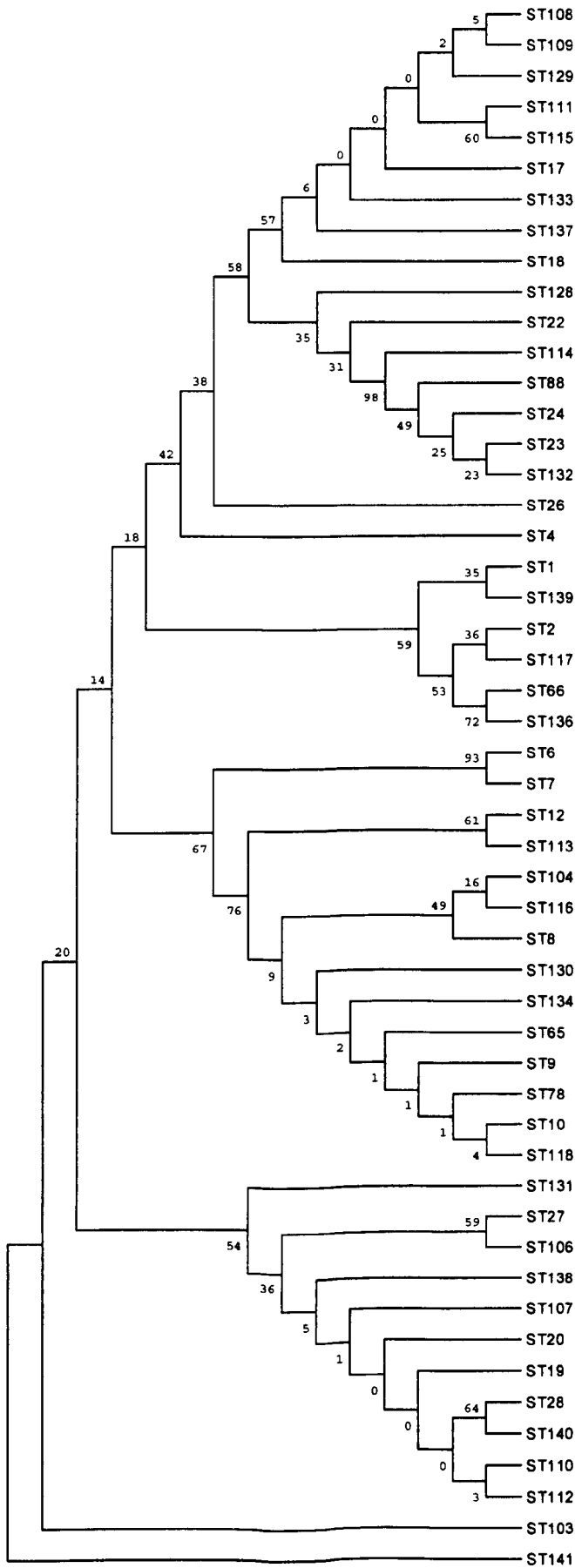


Figure 7.1: Maximum parsimony tree of 51 STs of invasive and carried GBS. Bootstrap values associated with branches are indicated.

Figure 7.2: Comparison of maximum parsimony tree (left) and neighbour joining tree (right) for concatenated sequences of 51 STs of carried and invasive GBS.

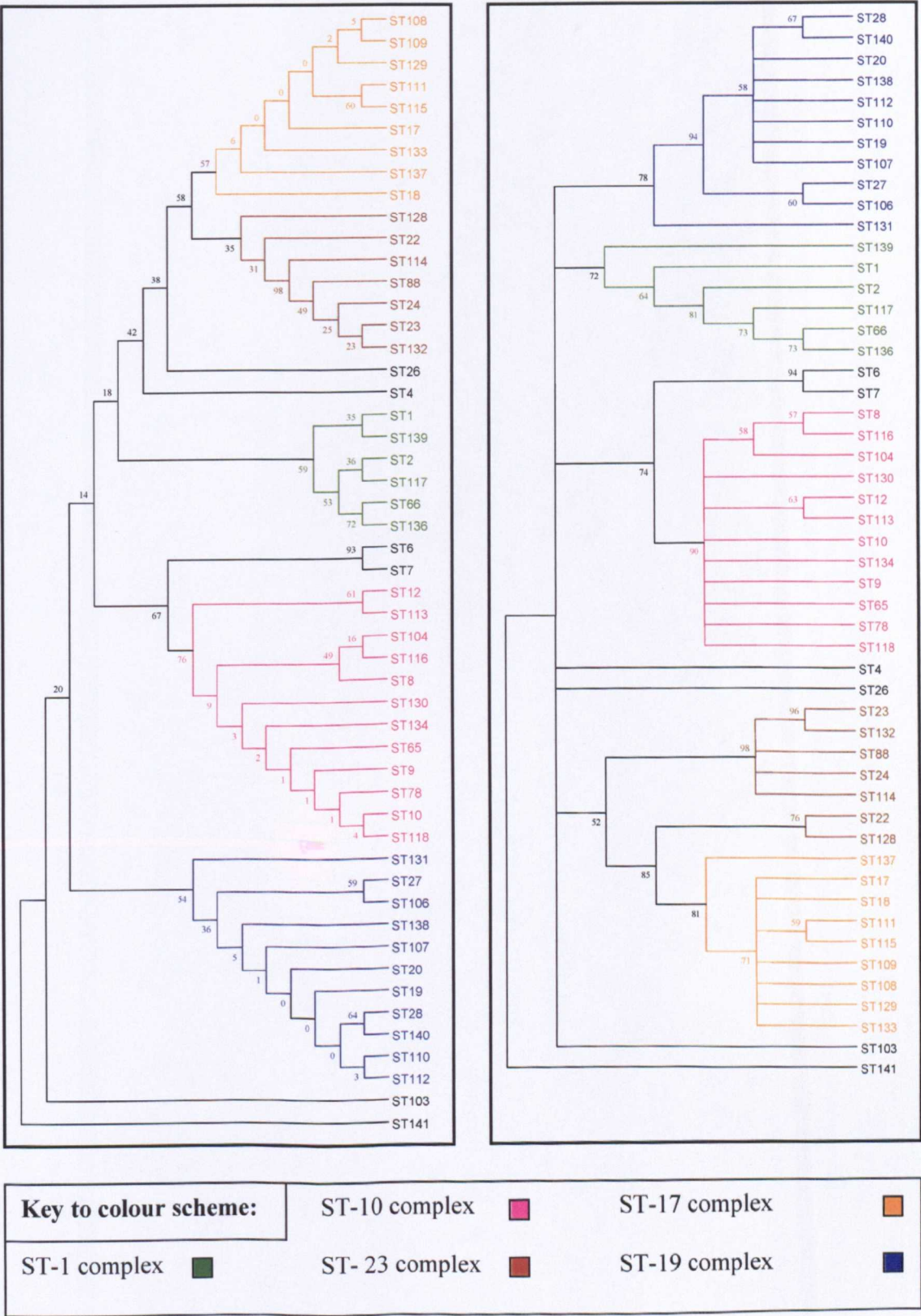
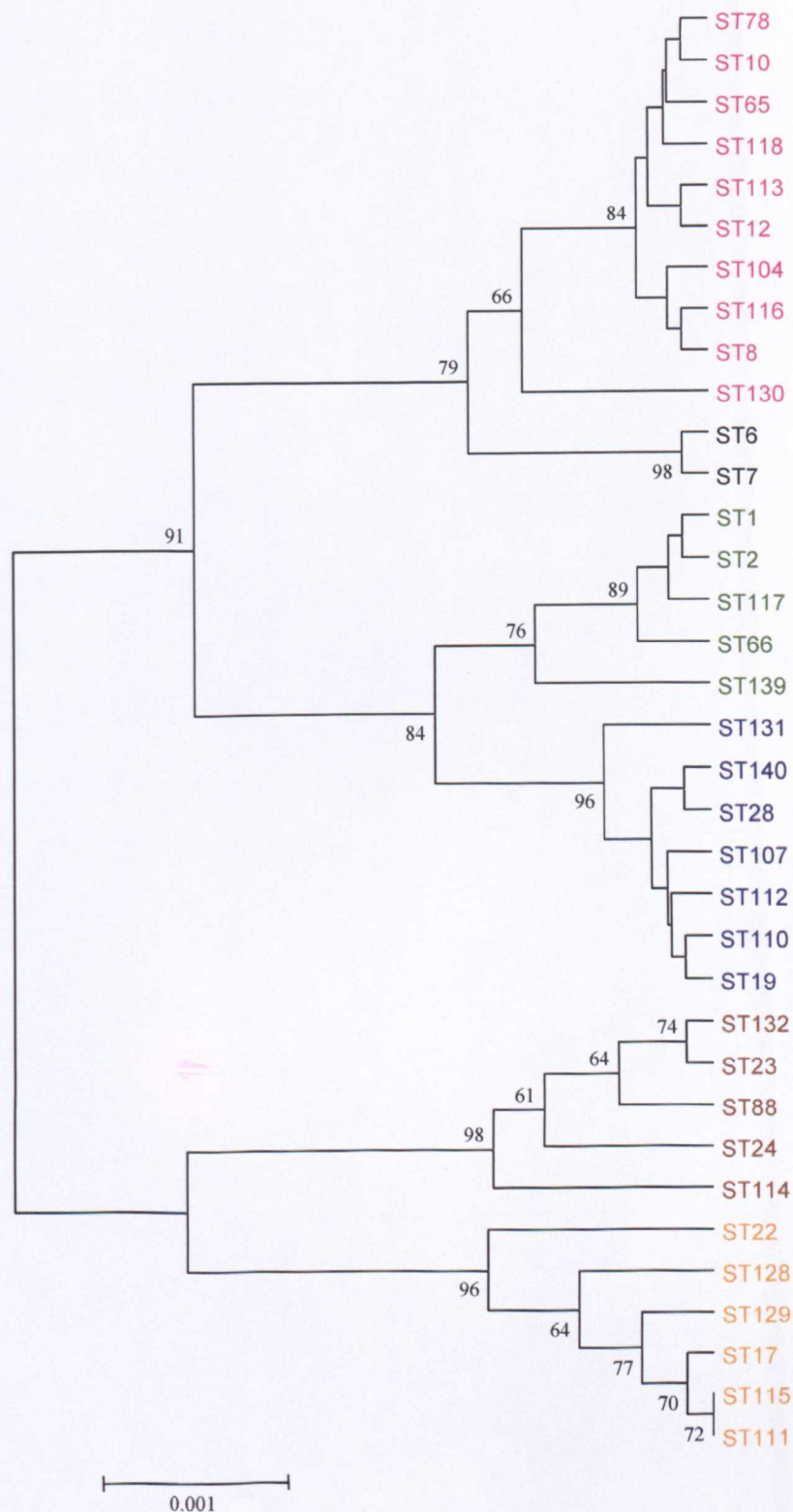


Figure 7.3: UPGMA tree for concatenated sequences of 47 STs of invasive and carried GBS. Long branches ST-4, 26, 103, 141 have been pruned. Bootstrap values are indicated. Colour scheme, as for Figure 7.2.



7.4.iii.c *Split decomposition analysis.* Split decomposition has been used to attempt to resolve relationships between the 7 clusters shown by the maximum parsimony and neighbour joining tree. The tree produced from the entire dataset is shown in Figure 7.4 shows a bipolar distribution of STs with ST-17 complex, ST-19 complex, ST-23 and ST-1 complex at one pole and ST-10 complex at the other pole. At nodes where multiple STs were located together, trees were formed with only those STs represented to indicate the relationships at those nodes.

The long branches ST-4, 26,103,141 were removed from the dataset and a further tree formed. This analysis (Figure 7.5) shows the population best represented by a network. ST-1 complex and ST-19 complex cluster together as do ST-23 complex and ST-17 complex. It can also be seen that ST-130 is distorting the network and as it is only represented by two isolates in the collection it is likely to be a new recombinant. This branch has been removed and the data reanalysed. Figure 7.6 shows the resultant tree. This time, the same clusters are revealed, but the relationships between the clusters are complicated and better represented by a network connecting the six clusters, rather than a branching pattern.

Figure 7.4: Tree formed by split decomposition of 51 STs of invasive and carried GBS. The boxes show the relationships of STs clustered together at the central nodes of the tree.

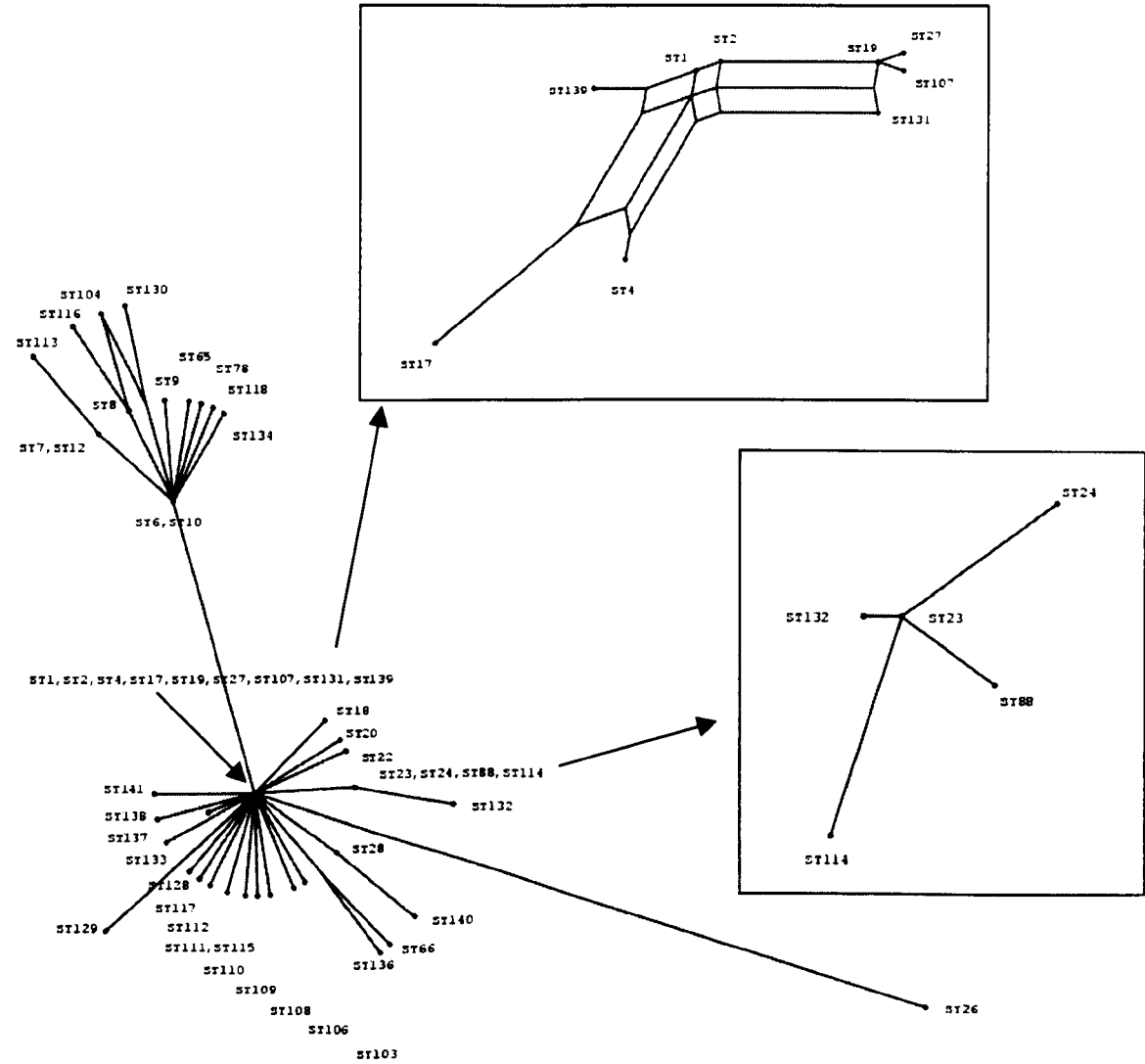


Figure 7.5: Tree formed by split decomposition for 47 STs of invasive and carried GBS. Long branches ST-4, 26, 103, 141 have been pruned. The position of ST-130 is indicated by a box.

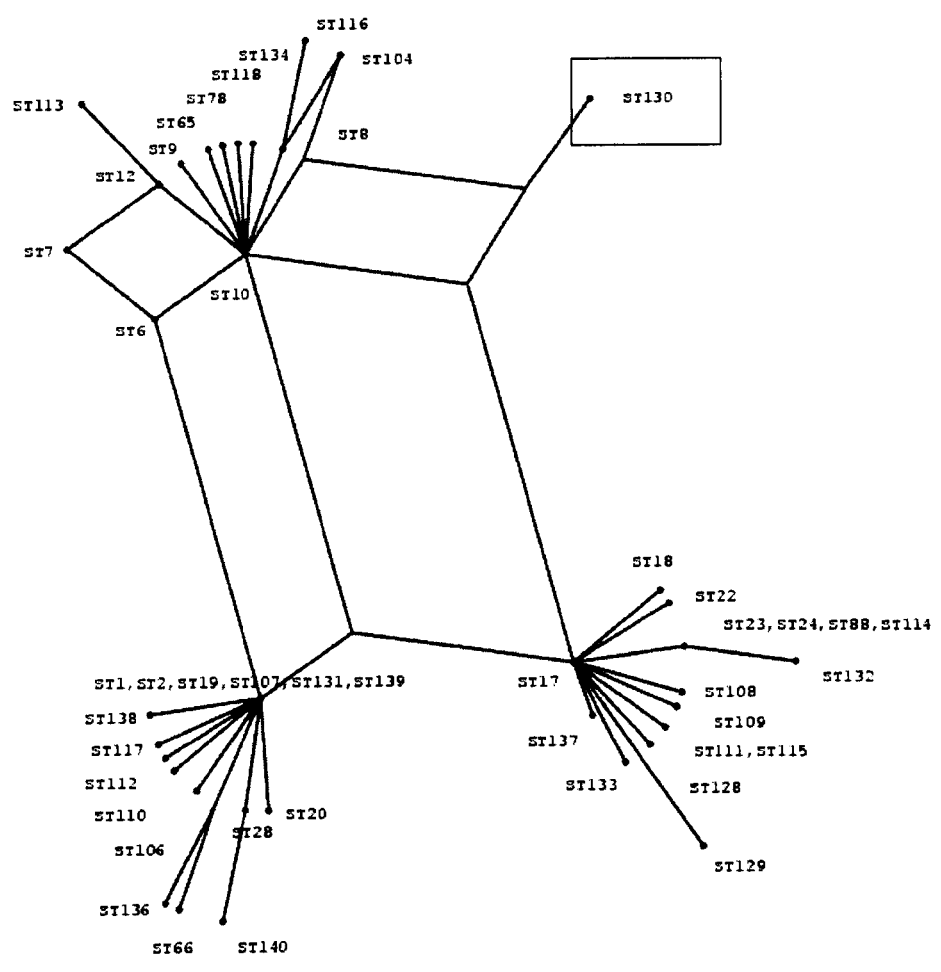
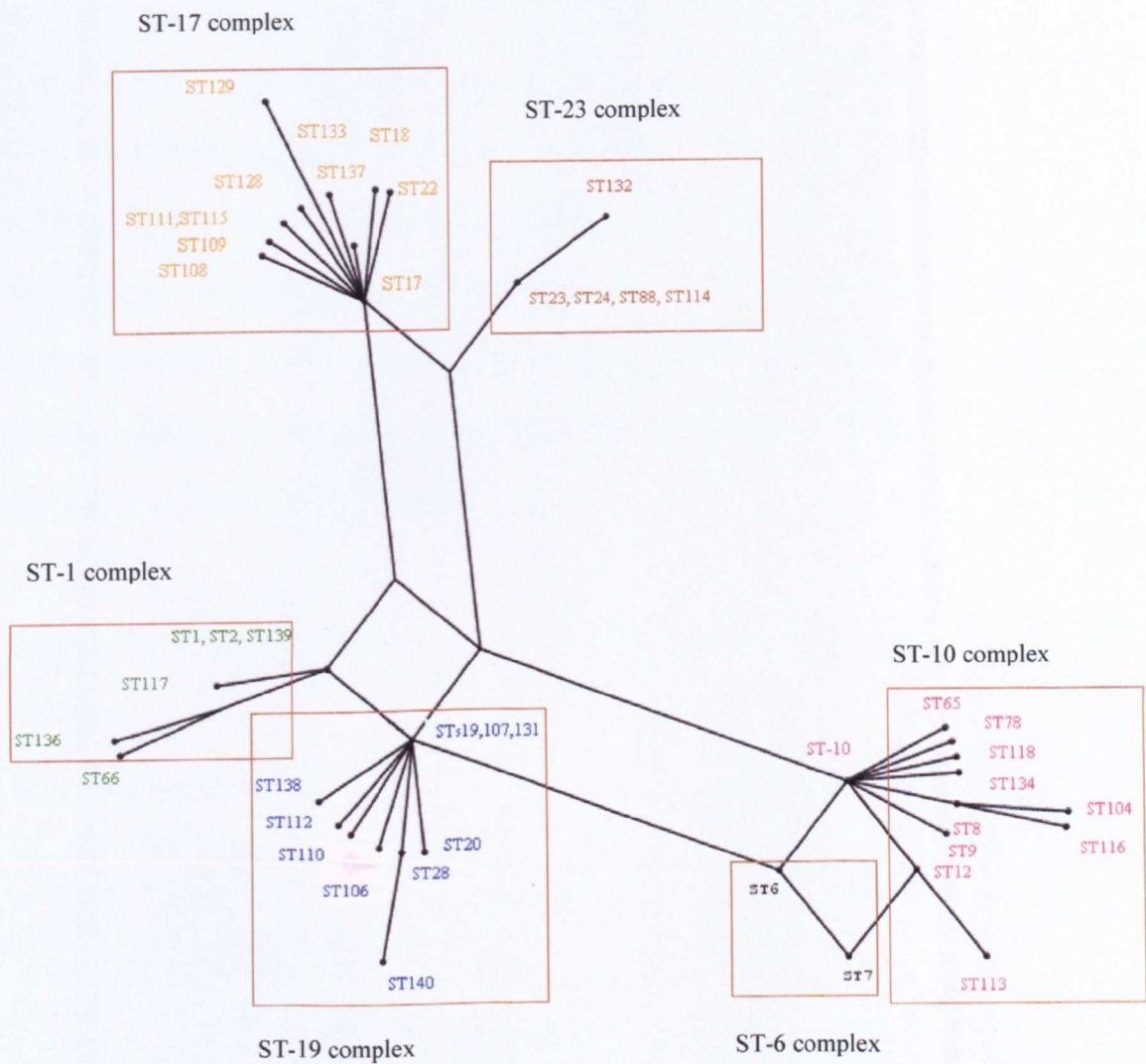


Figure 7.6: Tree formed by split decomposition for 46 STs of invasive and carried GBS. Long branches ST-4, 26, 103, 141 and 130 have been pruned. The boxes show the relationships of STs that are clustered together at nodes of the tree. Colour scheme as indicated in Figure 7.2.



7.4.iv *Diversity in the studied populations:*

Diversity was examined using concatenated sequences of the 51 STs which represent the collection of 299 isolates. Thirty-eight STs of the carried population were compared with 28 STs of invasive isolates. It can be seen that the diversity was approximately the same in the two populations in that there are an average of 5 nucleotide changes per 1000 base pairs of DNA ($\pi = 0.005$) in both carried and invasive populations of GBS. The segregating site (S) count and the number of pairwise differences (Π) are also similar (Table 7.3). Simpson's Index of diversity and 95% CI for the carried population was 0.89 (87.4 – 91.5) and was lower for the invasive population at 0.86 (81.7 – 90.3). It can be seen that the 95% CI for the two populations are overlapping which suggests that although there is a tendency for the carried population to be more diverse, this finding is not significant in this dataset.

Table 7.3: Comparison of Carried and Invasive GBS using tests of nucleotide diversity.

Test of Genetic Diversity	Carried	Invasive
Number of Sequences (n)	38	29
Length of Sequences (L)	3456	3456
Number of Segregating sites (S)	72	67
Segregating sites per site (S/L)	0.02	0.02
Nucleotide diversity ($\pi = \Pi/L$)	0.005	0.005
Average number of pairwise nucleotide differences (Π)	18.5	17.9
Simpson's index of diversity (D)	0.89	0.86

Average pairwise nucleotide differences between STs (Π) were calculated for the major clonal complexes seen in the dataset. Pairwise differences (Π) ranged from 1.0 to 6.0 within the clonal complexes (Table 7.4).

Table 7.4: Average number of pairwise differences (Π) within clonal complexes
 \pm Standard error of the mean (S.E.M).

Clonal complex	Π (\pm S.E.M)
ST-1 complex	3.7 (\pm 1.2)
ST-6 complex	1.0 (\pm 1.2)
ST-10 complex	2.2 (\pm 0.7)
ST-17 complex	3.7 (\pm 0.8)
ST-19 complex	2.6 (\pm 0.8)
ST-23 complex	6.0 (\pm 1.8)

Average pairwise nucleotide differences between the major clonal complexes were then calculated (Table 7.5). It can be seen that the number of pairwise differences was higher for STs located in different clonal complexes than for those located in the same clonal complexes. In other words, diversity within the clonal complex is lower than that seen between any two of the clonal complexes. It can also be seen that differences in the values of pairwise differences are not as marked between two areas of the tree where clonal complexes cluster more closely, in particular between ST-19 complex and ST-1 complex and also with ST-10 complex and ST-6 complex

Table 7.5: Average number of pairwise differences (Π) between clonal complexes, \pm Standard error of the mean (S.E.M).

Clonal complex	ST-1c	ST-6c	ST-10c	ST-17c	ST-19c
ST-6c	13.2 \pm 2.8				
ST-10c	20.9 \pm 3.5	8.5 \pm 2.6			
ST-17c	19.9 \pm 3.7	26.1 \pm 4.5	23.8 \pm 4.3		
ST-19c	10.3 \pm 2.5	12.8 \pm 3.3	19.9 \pm 3.9	25.6 \pm 4.4	
ST-23c	31.6 \pm 5.5	30.9 \pm 5.0	27.8 \pm 4.8	20.1 \pm 4.4	25.4 \pm 4.6

II

7.4.v *Association of STs and clonal complexes with invasiveness in*

neonates: ST-17 proved to be the only ST that was significantly associated with invasive disease in neonates ($p = 0.00002$, Table 7.6)). This finding was particularly true of late-onset neonatal infection, where ST-17 was associated with an odds ratio of 6.0 (Table 7.7). No ST was significantly associated with carriage. Following the grouping of STs into clonal complexes, it can be seen that again, ST-17 clonal complex was strongly associated with neonatal invasive disease (Table 7.8) and ST-23 complex was significantly associated with carriage in this analysis. ST-1 complex, ST-10 complex and ST-19 complex showed no association with either carriage or invasive disease. ST-19 complex, can be seen to be different to ST-17 complex since despite having a majority of serotype III isolates, it is not significantly associated with invasive disease. Capsular serotype III was significantly associated with invasive disease (OR 3.4 (2.0-5.7), p -value 0.000002).

Table 7.6: Calculation of invasiveness of STs, as measured by odds ratios (OR) with 95% CI and level of significance.

ST	No. invasive(%)	No. carried (%)	OR	95% CI	P-value
23	13 (11.9)	37 (19.5)	0.6	0.3-1.2	0.1
1	17 (15.6)	33 (17.4)	0.9	0.4-1.7	0.8
19	12 (11.0)	28 (14.7)	0.6	0.3-1.6	0.5
17	33 (30.3)	19 (10.0)	3.9	2.0-7.7	0.00002
8	5 (4.6)	12 (6.3)	0.7	0.2-2.3	0.7
12	1 (0.9)	10 (5.3)	0.2	0-1.3	0.06 *

Yates corrected test of significance used, except * Fisher exact test used.

Table 7.7: Calculation of invasiveness, as measured by odds ratios, of clonal complexes of GBS for early and late onset neonatal invasive disease. Ninety five percent CI and level of significance are listed.

ST	Early onset neonatal sepsis				Late onset neonatal sepsis			
	No.(%)	OR	CI	P-value	No.(%)	OR	CI	P-value
23	10 (15.6)	0.8	0.3-1.7	0.6	3 (6.7)	0.3	0.1-1.1	0.07
1	13 (20.3)	1.2	0.6-2.6	0.7	4 (8.9)	0.5	0.1-1.5	0.2
19	7 (10.9)	0.7	0.3-1.8	0.6	5 (11.1)	0.7	0.2-2.1	0.7
17	15 (23.5)	2.8	1.2-6.2	0.01	18 (40.0)	6.0	2.6-13.8	0.000002
8	4 (6.3)	1.0	0.3-3.5	0.6*	1 (2.2)	0.3	0.1-2.6	0.5*
Total	64				45			

Yates corrected test of significance used, except * where Fisher exact test used.

Table 7.8: Calculation of invasiveness, as measured by odds ratios, of clonal complexes of GBS neonatal invasive disease. Ninety five percent CI and level of significance are listed.

Clonal Complexes	No. Invasive (%)	No. Carried (%)	OR	95% CI	P- value
ST-17 complex	40 (36.7)	28 (14.7)	3.4	1.9-6.1	0.00003
ST-23 complex	14 (12.8)	44 (23.2)	0.5	0.2-1.0	0.004
ST-10 complex	13 (11.9)	38 (20.0)	0.5	0.2-1.1	0.1
ST-1 complex	21 (19.3)	38 (20.0)	1.0	0.5-1.8	1.0
ST-19 complex	19 (17.4)	38 (20.0)	0.8	0.4-1.6	0.7

The population of GBS studied represents a collection of almost 300 isolates assembled from a defined geographical area of the UK over a similar time period. The purpose of the study was to use this collection, which represents the ecology of GBS in the setting of neonatal invasive disease, to examine the population biology of GBS.

Analysis of population structure has shown that there are 6 major clusters of GBS, all of which contain isolates from carriage and invasive disease. Three different methods were used to demonstrate this pattern of clustering, UPGMA, split decomposition and calculation of pairwise differences (II). The major complexes seen were ST-1 complex, ST-10 complex, ST-17 complex, ST-19 complex, ST-23 complex and ST-6 complex.

In order to define relationships between the complexes, long branches were removed and subsets of strains analysed. Using the tree building methods of neighbour-joining and maximum parsimony some of the major branches of the trees were associated with low bootstrap values. This finding suggests that there is conflict within the tree, that is, the branching pattern of the tree has been distorted by the presence of recombinational events. By eliminating long branches of more distantly related strains and reconstructing the UPGMA tree, the bootstrap values associated with the major complexes were all above 70% supporting their identity as clonal complexes.

A further possibility is that the branching tree structure is not able to fully describe the relationships between the major complexes. The split decomposition method was used as an alternative way to demonstrate relationships between clusters. The resultant network revealed a similar pattern with the same 6 major complexes, but a network rather than branching lines interconnecting these complexes. This is consistent with the presence of recombinational events occurring in the population of GBS that are interfering with a tree like branching structure of the population.

Measures of diversity, were calculated to determine whether the clonal complexes described by the trees can be supported numerically. The amount of measured diversity within and between complexes supports the identification of ST-23 and ST-17 as clonal complexes. The situation was more ambiguous with ST-6/ ST-10 complexes and ST-19/ ST-1 complexes, in that, although the diversity was greater between than within the complexes, the difference was not as great as for ST-17 and ST-23 complexes.

Epidemiological data were then combined with the phylogenetic results. ST-17 is significantly associated with neonatal invasive disease and was represented in the invasive collection three-fold more frequently than other STs. This finding was unique, in that no other ST was significantly associated with either invasive disease or carriage. In particular, ST-19, which shares the same capsular serotype (III) as ST-17, was not associated with invasive neonatal disease. These findings were confirmed when the data were reanalysed using complexes rather than individual STs. The association of ST-17 complex with invasive disease

was maintained and ST-23 complex proved to be significantly associated with carriage.

Capsular serotyping revealed that ST-17 complex isolates were serotype III. None of the other major STs were uniquely associated with a single capsular serotype, ST-1 tended to be V and similarly, ST-8 was associated with IB, ST-19 with III and ST-23 with IA. That a sequence type can have more than one serotype suggests that there may be recombination occurring across the capsular locus. This has not been shown to occur *in vivo*, however.

Serotype III is numerically most prevalent in both carried and invasive isolates and was significantly associated with invasive disease. Whether this association is due to the presence of ST-17 complex isolates within type III isolates seems likely although the numbers are too small to adequately investigate heterogeneity of ST invasiveness within the same capsular type. It can be seen that ST-17 complex type III isolates are associated with invasive disease whereas ST-19 complex type III isolates are not. This suggests that the invasiveness of ST-17 complex isolates may be independent of capsular type III and that another factor may be responsible for virulence of this clonal complex in neonates.

Several interesting observations can be made from these analyses of the population structure of GBS. There are a number of successful clonal complexes of GBS responsible for human carriage and invasive disease. One complex (ST-17 complex) is hypervirulent in neonates. The remaining complexes are

successful well-adapted complexes, as indicated by their prevalence in the population of both carriage and invasive isolates.

Recombinational events occur in GBS allowing isolates with identical allelic profiles to have different capsules and resulting in distortion of the tree like structure of GBS.

The association of invasive disease with ST-17 complex was particularly strong for late onset neonatal disease. This is an important finding, especially since the introduction of intrapartum prophylaxis to women has proved to have no effect on prevalence of late onset neonatal disease. Further study of this hypervirulent clone of GBS may contribute to the understanding of the pathogenesis of neonatal GBS disease.

Chapter 8: Invasive GBS disease in human cases of age greater than 60 years.

8.1 AIMS:

To investigate GBS invasive infection in adults over 60 years, as this age group is at increased risk. Clinical presentation was recorded and isolates were characterised by MLST to determine the population of GBS responsible for disease in this setting and to see whether hypervirulent clones of GBS could be defined.

8.2 INTRODUCTION:

The rate of invasive GBS disease has generally increased over the last few decades⁹². More than two-thirds of GBS disease in the USA occurs in adults⁹¹. GBS infections commonly affect those with underlying medical conditions, including diabetes mellitus, liver cirrhosis, malignancy, vascular disease and decubitus ulcers⁹². Studies have suggested that 70-93% of adult cases of GBS infection had one or more underlying medical illness^{62,93,94}. Indeed, the increasing rate of GBS disease may be attributed, in part, to an expanding population of adults who are living longer with significant medical co-morbidities.

Clinical manifestations of GBS in adults are numerous and varied. Common diagnoses include skin and soft tissue infection, bone and joint (native and

prosthetic) infection and pneumonia. Less frequently, endocarditis, meningitis and endophthalmitis are the presenting syndromes⁹¹.

Serotype distribution of GBS isolates indicates that type IA, III and V are common in the adult population¹⁴⁹ and is an important factor to consider in the planning of a GBS vaccine strategy aimed at elderly patients.

The mean age of non-pregnant adults with invasive GBS disease is 60 years and elderly adults in nursing home accommodation appear to be at particular risk⁹⁵. Annual incidence rates of GBS disease in adults have been estimated in the USA as 4.1 to 7.2/100,000 population⁹⁵. Figures from the UK have not been reported in recent times.

8.3 MATERIALS AND METHODS:

8.3.i *Isolate collection:*

GBS isolates were collected in the Oxford area from participating hospitals as described in Chapter 3. Only GBS isolates from cases aged 60 years or over were included for study (n=70).

Age at onset of infection was recorded for each case and where the case records were available for examination (JRH) clinical diagnosis was listed.

8.3.ii *MLST methods:*

MLST was carried out on the collection as described in Chapter 2.

8.4 RESULTS:

Seventy cases of invasive GBS disease in patients > 60 years were identified from the enhanced surveillance study. The age distribution of cases is depicted in Figure 8.1 and 8.2. Case records were retrieved from 26 patients, who had been admitted the Oxford Radcliffe Hospitals. Admitting diagnosis was recorded for these cases and is indicated in Table 8.1. The majority of cases (16/26, 61.5%) presented with skin, soft tissue or bone and joint (native or prosthetic) infection.

Figure 8.1: Number of cases of invasive GBS disease according to age group.

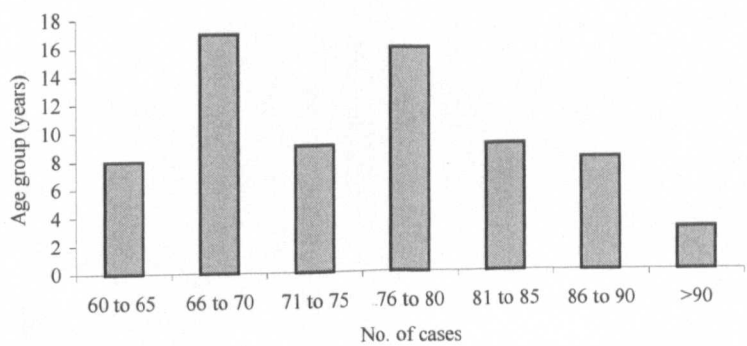


Figure 8.2: Numbers of invasive GBS cases from adults in Oxfordshire in age groups, 60 to >90 years. Total number of cases (columns, with error bars) and age- adjusted rates (lines) are indicated.

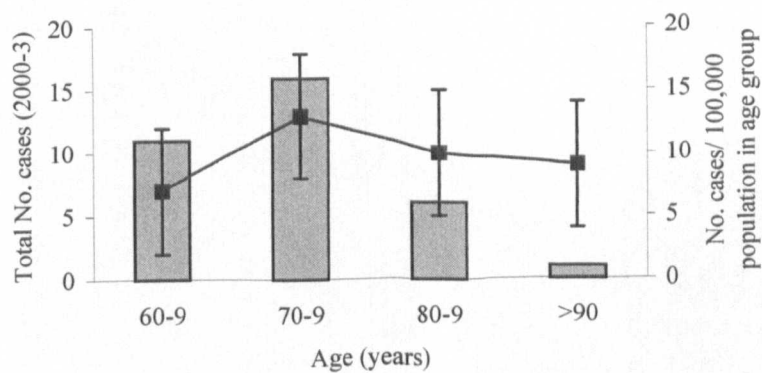


Table 8.1: Clinical diagnosis of cases of invasive GBS at age > 60 years.

Clinical diagnosis	Number
Skin and soft tissue	6
Native bone or joint infection	6
Prosthetic joint infection	4
Bacteraemia, no focus	5
Pneumonia	1
Infective endocarditis	2
Urinary tract sepsis	1
Abdominal infection	1

Twenty-seven STs were identified in the collection. Clustering of STs, based upon concatenated sequences, is shown in Figure 8.3, by the algorithm of UPGMA and Figure 8.4, using split decomposition.

The clonal complexes identified previously (Chapter 7) were again seen. Six major complexes were present on both trees (UPGMA and SplitsTree). The most frequently identified complex was ST-1 complex, which accounted for 17 isolates (24.3%). ST-19 complex, ST-10 complex and ST-23 complex were also commonly seen. Fewer isolates fell into ST-17 complex (n=6) and ST-6 complex (n=3). When the population of isolates from invasive GBS disease in cases >60 years was compared with that of asymptomatically carried GBS from pregnant women, it can be seen that no clonal complex was significantly associated with disease in cases > 60 years (Table 8.2) and the odds ratios were all close to 1.0.

Table 8.2: Numbers (%) of STs isolated from invasive disease in age >60 years compared with asymptotically carried isolates, with p-values.

Clonal Complex	Invasive >60 yrs Number (%)	Carried isolates Number (%)	Odds ratios (95% CI)	P-value*
ST-23 complex	11 (15.7)	44 (23.2)	0.6 (0.3-1.3)	0.3
ST-10 complex	15 (21.4)	38 (20.0)	1.1 (0.5-2.2)	0.9
ST-1 complex	17 (24.3)	38 (20.0)	1.3 (0.7-2.6)	0.6
ST-19 complex	14 (20.0)	38 (20.0)	1.0 (0.5-2.1)	0.9

* Yates-corrected test of significance
Abbreviations: CI, 95% confidence intervals.

Figure 8.3: UPGMA tree with bootstrap values for concatenates sequences of STs identified in collection of isolates of invasive GBS from adults >60 years (Numbers in brackets indicate the number of isolates identified as that ST, except where blank where n=1).

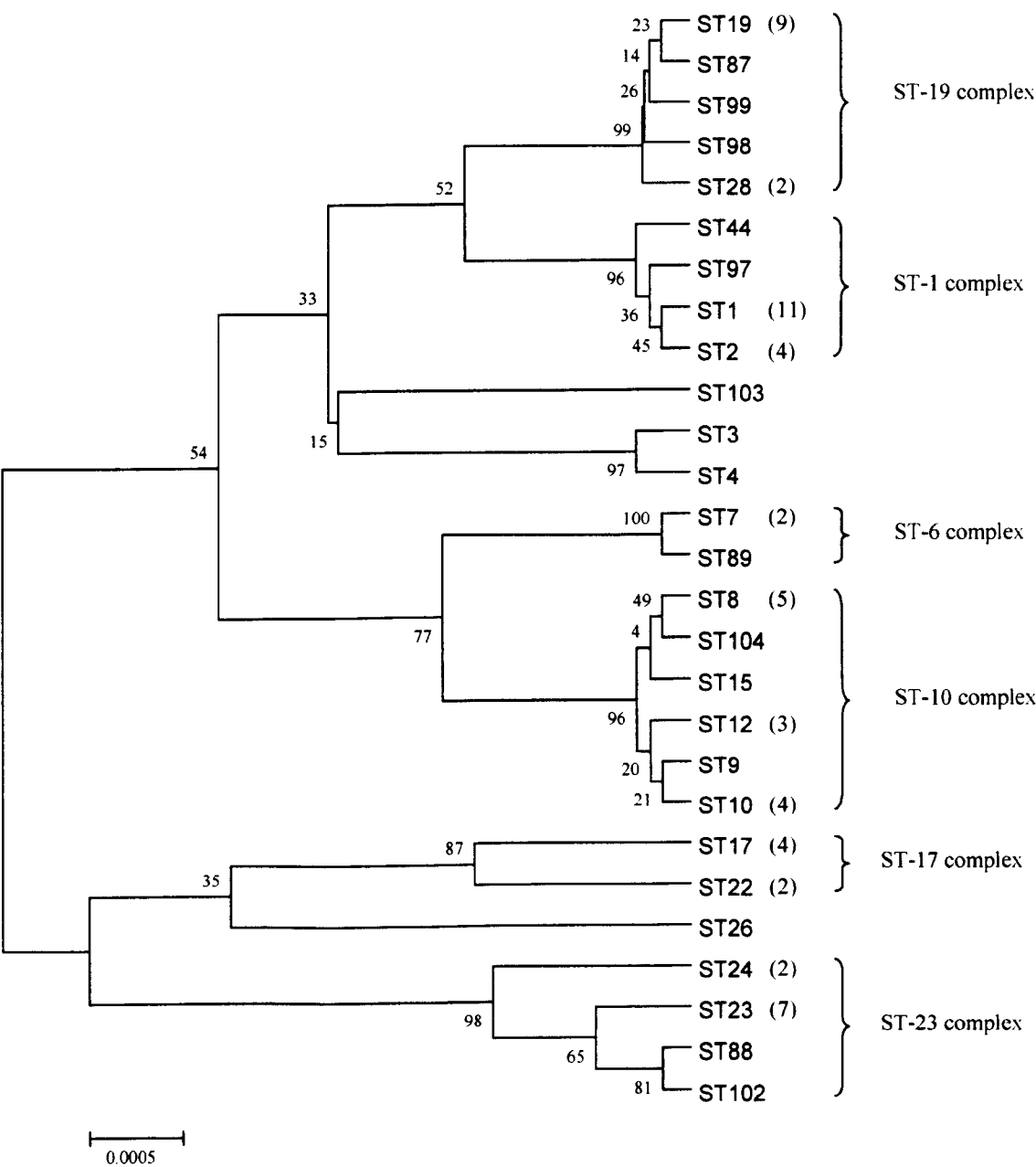
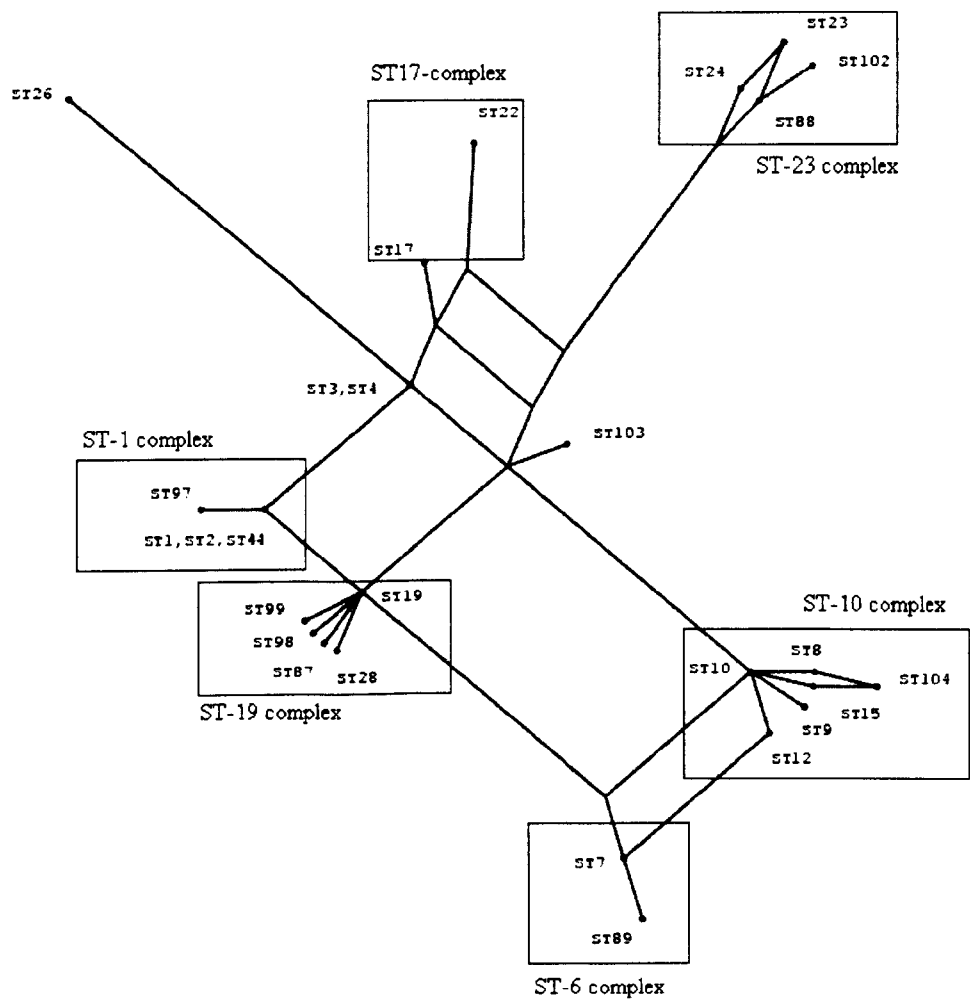


Figure 8.4: SplitsTree diagram for STs identified in collection of isolates of invasive GBS from adults >60 years.



8.5 DISCUSSION:

This study is an investigation of invasive GBS infection in older adults (>60 years) in the Oxford area. A sample of 70 isolates were studied. Invasive GBS disease is commonly seen in this group, with clinical presentation frequently involving skin, soft tissue or osteoarticular infection.

MLST was carried out on the isolates and showed that there are four important clonal complexes accounting for more than 80% of the invasive GBS disease seen in the adults > 60 years. These are ST-1 complex, ST-10 complex, ST-19 complex and ST-23 complex. The clustering seen using the methods of UPGMA and split decomposition is similar to that seen in neonatal invasive GBS and carried GBS (Chapter 7).

It can be seen, from comparison with a collection of isolates isolated from asymptomatic carriage in the same geographical area that there is no significant difference between the two groups. Clonal complexes that are prevalent in asymptomatic carriage are also successful as pathogens in the setting of invasive GBS disease in the adults > 60 years. This is in contrast to the case of neonatal GBS infection, where ST-17 complex is hypervirulent. ST-17 is uncommon as a pathogen in the adults > 60 years.

The collection of carried isolates from pregnant women may not be the most representative of carriage in the adult population over 60 years of age. Future

studies utilising collections of carried isolates from this age group may be more appropriate.

It can be concluded, from this investigation, that successful lineages of GBS identified in carriage in pregnant women are also the most prevalent lineages associated with invasive GBS disease in older adults and that hypervirulent clones have not been identified.

Chapter 9: Relationship between bovine and human group B streptococcus.

This chapter has been published as:

Bisharat, N., Crook, D.W.M., Leigh, J., Harding, R.M., Ward, P.N., Coffey, T.J., Maiden, M.C., Peto, T., Jones, N. Hyperinvasive Neonatal Group B Streptococcus Has Arisen From A Bovine Ancestor. *Journal of Clinical Microbiology* 2004; 42(5): 2161-2167 (Appendix 3).

9.1 AIMS:

To investigate the relationships of human and bovine GBS isolates using MLST data. In addition, to determine whether human and bovine GBS are distinct populations, or whether there is some overlap between the two populations given the link suggested by epidemiology between the two populations. Lastly, to examine possible evolutionary scenarios for the recent emergence of human-pathogenic GBS and their relationship to bovine GBS.

9.2 INTRODUCTION:

GBS was initially described as an animal pathogen causing bovine mastitis in 1887¹⁵⁰. Human infections caused by this bacterium were only reported 50 years later in the 1930s^{1,151}. Neonatal disease was rarely reported until 1960s when numerous reports linked neonatal infections with this organism^{2,3,152}, and by the 1970s GBS had become the leading neonatal pathogen^{54,105,153}. The reasons behind the rapid and sustained emergence of GBS neonatal disease have not been completely elucidated. A possible explanation has been association with bovine GBS^{98,154}, but most studies have concluded that the human and the bovine populations are distinct and unrelated^{155,156}.

9.3 MATERIALS AND METHODS

9.3.i *Bacterial isolate collections.*

The bovine isolates (n=111) were obtained from milk samples of cows with evidence of clinical mastitis. Twenty-five isolates were provided by the Institute of Animal Health (IAH), Compton, Berkshire, UK. Twelve of these had been collected by the Central Veterinary Agency at Weybridge, Surrey, UK, during the mid 1950s. The remaining isolates (n=13) were collected and supplied during 1991-1992 by the Milk Marketing Board to the IAH. A further 86 isolates were purchased from the Veterinary Laboratories Agency (VLA), Bury St Edmunds, Suffolk, UK. These had been collected between 1987-1996 from farms around the UK and represented a collection of diverse geographical origin. Each isolate was a single isolation from an individual cow within a herd and additional isolates were not collected from the same herd. For interest, four disease-causing isolates collected from other animals (elephant, dog (n=2), and goat) were included, which were supplied by the VLA.

The human isolates comprised 152 invasive and carriage isolates from the UK, USA, Japan, New Zealand, Thailand, Singapore, and Israel, which were characterized in a previous study and represent the global collection described in Chapter 6.

9.3.ii *Identification and characterization of isolates.*

Methods described previously for the isolation of isolates, DNA extraction and MLST, were followed. Capsular serotyping was carried out using the latex agglutination method.

9.4 RESULTS:

9.4.i *Genotypes identified.*

Fifty STs were represented in the whole collection, of which 26 were identified in humans only, 17 were unique to the bovine isolates and 3 were present in isolates from both humans and bovines. The remaining 4 STs were associated with isolates from other animals, two from dogs and one each from an elephant and a goat. The most common STs were ST-67 accounting for 73 of the 111 (65.8%) bovine isolates and ST-17 that was found in 44 of the 152 (29.0%) human isolates. A total of 31 STs were identified only once in this isolate collection. The characteristics of the genotypes identified in the dataset are shown in Table 9.1.

9.4.ii *Capsular serotyping.*

Seventy six percent of the bovine isolates were non-typeable (Table 9.1). For the typeable isolates, serotype II was the most common (n=22), the remaining four isolates were characterized as serotype III (n=3) and serotype 1b (n=1). In contrast, only 3.3% of the human isolates were non-typeable. The majority of human isolates belonged to serotype III (51%). Eight isolates (5.3%) from the human collection belonged to serotype II.

Table 9.1: Characteristics of Main GBS Sequence Types according to Serotype, Host, and Disease State.

ST	Allelic profile*	No.of isolates in ST	Serotype (No. of isolates)	Host (No.of isolates)	Disease state (No. of isolates)
67	13.1.1.13.1.1.5	73	II (14). IB (1). NT (58)	B (73)	BM (73)
17	2.1.1.2.1.1.1	44	III(44)	H (44)	NI(33). AC(9). AI(2)
1	1.1.2.1.1.2.2	21	V(9). VIII(4). VI(4). III(2). IB(1). NT(1)	H (21)	AC (16). AI (3). NI (2)
19	1.1.3.2.2.2.2	21	III(17). II(2). V(1). NT(1)	H (20). B (1)	AC(14). NI(3). AI(2). NC(1). BM (1)
23	5.4.6.3.2.1.3	17	IA(11). III(4). V(1). NT (1)	H (16). B (1)	AC(7). NI(5). AI(3). NC(1). BM (1)
61	13.1.1.13.1.1.1	9	III (1). II (2). NT (6)	B (9)	BM (9)
2	1.1.3.1.1.2.2	8	II (7). NT (6)	B (6). H (2)	BM (6). AI (2)
8	4.1.4.1.3.3.2	7	IB (6). NT(1)	H (7)	AC(4). NI(1). AI(2)
10	9.1.4.1.3.3.2	5	IB(3). II(1). NT(1)	H (5)	AC(2). NI(1). AI(2)
11	9.3.7.1.3.3.2	5	III(5)	H (5)	AI(5)
76	13.1.1.13.1.14.1	5	II (1). NT (4)	B (5)	BM (5)
7	10.1.2.1.3.2.2	3	IA (3)	H (3)	AC (1). NI (2)
12	10.1.4.1.3.3.2	3	IB (3)	H (3)	NI(2). AC(1)
26	1.1.5.4.1.4.6	3	V(3)	H (3)	AC(2). NI(1)
28	1.1.3.5.2.2.2	3	II(3)	H (3)	AC(2). NI(1)
72	13.1.1.13.1.2.5	3	NT (3)	B (3)	BM (3)
4	1.1.4.1.1.3.4	2	IA (2)	H (2)	AC (1). NI (1)
15	9.1.4.1.5.3.2	2	IB(2)	H (2)	AI(1). NI(1)
22	13.3.1.3.1.1.1	2	II(2)	H (2)	AC(1). AI(1)

Table 9.1 continued

ST	Allelic profile*	No.of Isolates/ ST	Serotype (No. of isolates)	Host (No.of isolates)	Disease state (No. of isolates)
5	12,1,4,1,1,3,4	1	IA (1)	H (1)	AC (1)
6	9,1,2,1,3,2,2	1	IB (1)	H (1)	AC (1)
9	8,1,4,1,3,3,2	1	IB (1)	H (1)	AC(1)
13	11,3,4,1,3,3,2	1	VI(1)	H (1)	AC(1)
14	1,1,2,1,5,2,2	1	VI(1)	H (1)	AC(1)
16	1,1,4,1,6,3,4	1	IA(1)	H (1)	AI(1)
18	3,1,1,2,1,1,1	1	III(1)	H (1)	NI(1)
20	1,2,3,2,2,2,2	1	III(1)	H (1)	NI(1)
21	1,9,3,2,2,2,2	1	III(1)	H (1)	NI(1)
24	5,4,4,3,2,3,3	1	IA(1)	H (1)	AC(1)
25	5,4,6,3,8,1,3	1	III(1)	H (1)	NI(1)
27	1,1,3,4,2,2,2	1	III(1)	H (1)	AI(1)
29	2,1,1,8,1,1,1	1	III(1)	H (1)	NI(1)
30	1,1,8,2,2,2,2	1	V(1)	H (1)	AC(1)
62	13,1,1,13,11,1,5	1	II (1)	B (1)	BI (1)
63	13,1,1,2,1,10,1	1	NT (1)	B (1)	BI (1)
64	13,1,1,2,1,1,1	1	III (1)	B (1)	BI (1)
69	13,1,1,16,1,1,5	1	NT (1)	B (1)	BI (1)
73	13,1,1,13,14,9,5	1	NT (1)	B (1)	BI (1)
74	13,1,1,13,1,13,5	1	NT (1)	B (1)	BI (1)
75	13,1,14,13,1,1,5	1	NT (1)	B (1)	BI (1)
79	13,1,1,13,14,1,5	1	NT (1)	B (1)	BI (1)
80	19,1,1,13,1,1,5	1	II (1)	B (1)	BI (1)
81	1,12,3,2,2,1,2	1	NT (1)	D (1)	DI (1)
82	20,13,15,1,15,1,5	1	NT (1)	E (1)	EI (1)
83	2,1,1,2,14,1,1	1	III (1)	B (1)	BI (1)
84	10,1,2,1,3,1,2	1	NT (1)	D (1)	DI (1)
85	13,1,17,15,1,1,5	1	NT (1)	B (1)	BI (1)
86	1,1,3,2,2,1,2	1	NT (1)	G (1)	GI (1)
100	13,1,1,13,1,17,5	1	NT (1)	B (1)	BI (1)
101	13,1,1,13,1,18,5	1	II (1)	B (1)	BI (1)

Abbreviations: ST, sequence type; NT, non-typeable; A, human adult; B, bovine; M, mastitis; H, human; N, human neonatal; I, invasive disease; C, carried isolate.

* Allelic profiles for each gene are presented in the order, *adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, *tkl*.

9.4.iii *Mobile genetic elements within the glcK gene.*

PCR amplification of the internal fragment of the *glcK* gene in eight isolates of ST-67 from the bovine collection produced a 3.0 kb band instead of 0.6 kb band that was observed in the remaining isolates (Figure 9.1). This increase in band size was found to be due to a mobile genetic element, which was inserted at the identical point in the *glcK* gene in each of the eight bovine isolates. This mobile genetic element (2314 bp) contained one open reading frame (568 amino acids), and a BLAST search yielded an approximate 50% identity to group II intron reverse transcriptase. For the purposes of the analysis using concatenated sequences, the nucleotide sequence of the mobile element was deleted from the text of nucleotide sequences leaving the intact *glcK* allele.

Figure 9.1: Agarose gel (1%) demonstrating the PCR product (*glcK* gene) in two strains of GBS.

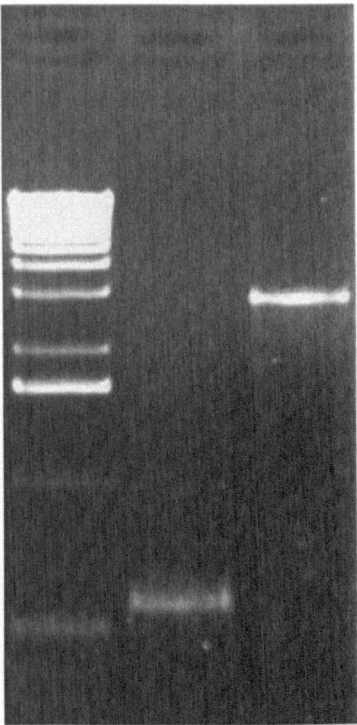
The first lane is the DNA size marker, the second shows a band of approximately 600 bp (control) and the third lane shows a band of approximately 3000 bp due to the presence of an insertion sequence.

Size of DNA
markers
(Base -pairs)

3054
2036
1636

1018

500

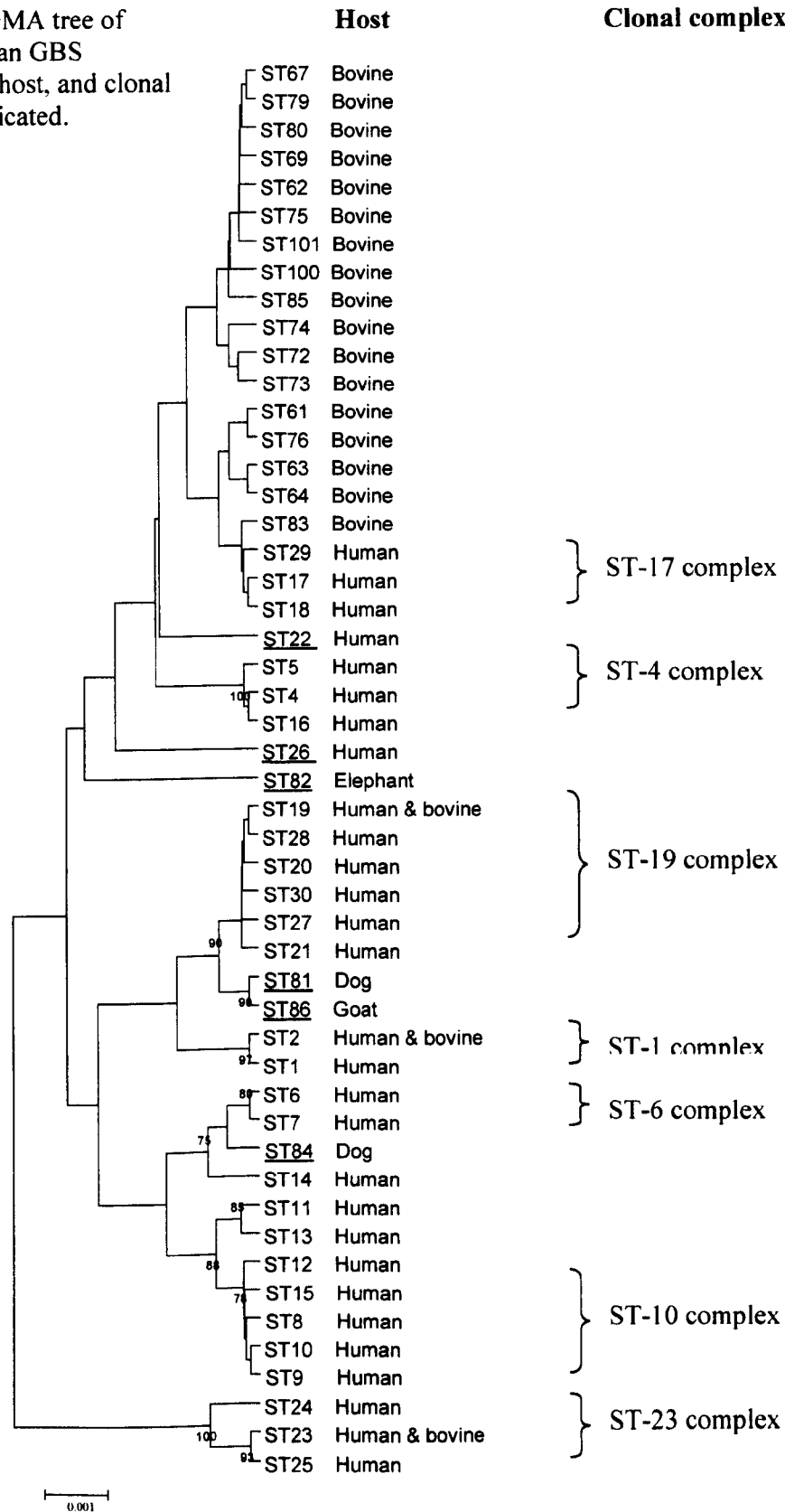


Marker Control Strain
 with
 insertion

9.4.iv *Relationship between bovine and human isolates.*

The seven clonal complexes of human strains as previously shown (Chapter 6) are evident in Figure 9.2. The current analysis uses the UPGMA clustering algorithm of MEGA, based on the DNA sequence of the concatenated alleles and shows that human ST-1 complex, ST-19 complex, ST-6 complex and ST-10 complex cluster together and separately from ST-17 complex and ST-23 complex (Figure 9.2). The bovine isolates are clustered together (n=103, (93%)). Human ST-17 complex, previously identified as having increased virulence in neonates¹⁵⁷, now groups within the main bovine cluster. Placement of human ST-4 complex as an “out group” of the bovine cluster rather than with the other human lineages is an apparent rearrangement compared with our previous analysis reflecting the different method of analysis based on concatenated sequences. A minority of bovine isolates (n=8, 7%) are found within the main human STs, ST-23, ST-19, and ST-2. Isolates from dog and goat cluster with the human STs. The elephant isolate and human ST-22 and ST-26 do not appear closely related to any of the clusters. Bootstrap values have been calculated and those greater than 70% are shown against the branches in Figure 9.2. Bootstrap values are percentages of 500 computer-generated trees produced by randomly sampling the sequences and are shown at the nodes. Underlined sequence types indicate sequence types to be pruned for further analysis.

Figure 9.2: UPGMA tree of bovine and human GBS collections. ST, host, and clonal complex are indicated.



The analyses thus far show that human ST-17 complex groups more closely with the bovine STs than with other human STs, but strong support for this conclusion is lacking, given the low bootstrap values at these branches. To further assess reliable structure within the tree the data were examined using split decomposition analysis, which allows for the fact that real evolutionary data may not be best described by a branching tree format, given that recombinational events may have occurred within the population.

The split graphs representation of the structure is shown in Figure 9.3 and indicates that several STs are distantly related to most others (underlined in Figure 9.2). As the algorithm gives undue prominence to these distant STs, better discrimination between bovine and human STs was obtained on the majority of the dataset by repeating the analysis after removal of 2 distant human STs (ST-22, ST-26) and the 4 STs from other species (dog (ST-81, ST-84), goat (ST-86), elephant (ST-82)).

Figure 9.4 shows that the bovine STs are in a separate split from the human STs. This bovine split also contains the human ST-17 complex (significantly associated with invasive neonatal disease). Other branches in Figure 9.4 show the same groupings of STs apparent in Figure 9.1. It can be seen that apparently clear phylogenetic relationships in the UPGMA dendrogram in Figure 9.2 are more complicated and are represented by a network, which has been discussed in Chapter 7. However while the presence of parallel splits in Figure 9.4 shows ambiguous phylogenetic relationships between clusters of mainly human STs,

the finding that the human ST-17 complex groups within a branch of bovine strains is a clear and robust result.

Figure 9.3: Tree drawn by split decomposition showing relationships between bovine and human GBS collections.

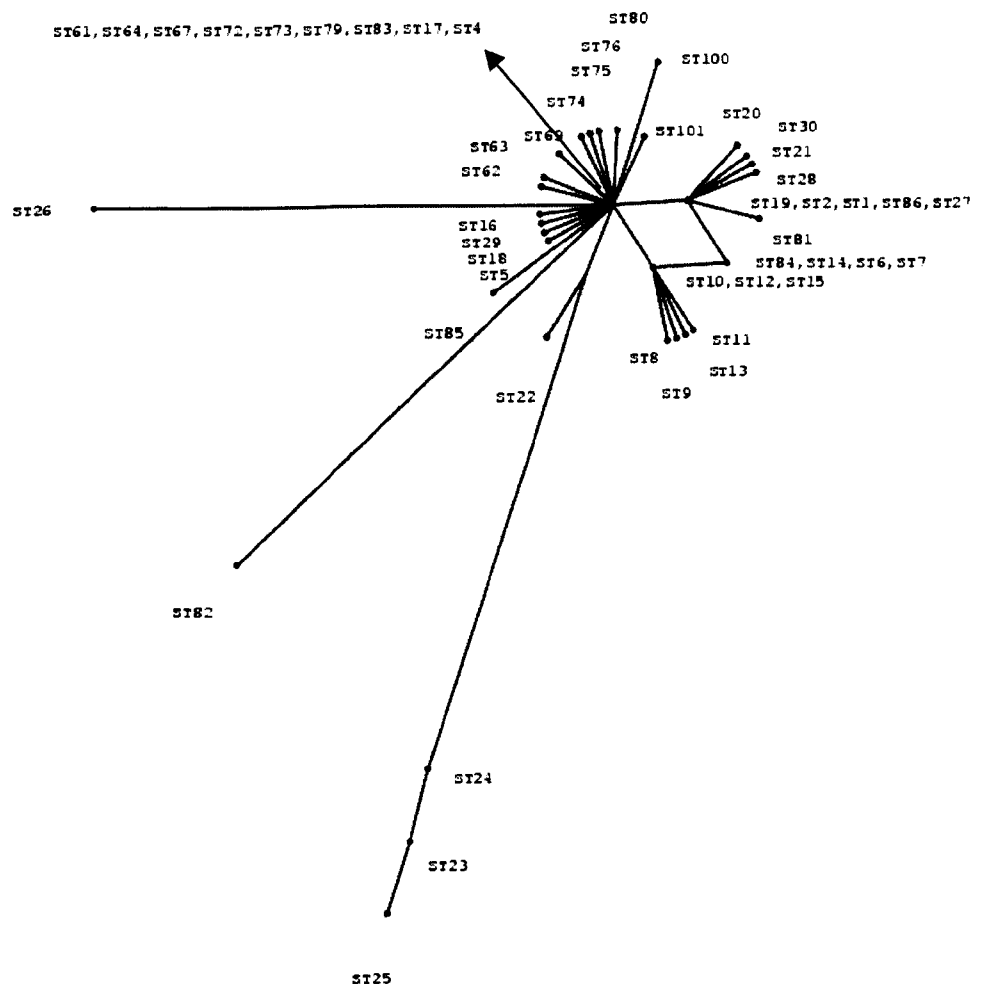
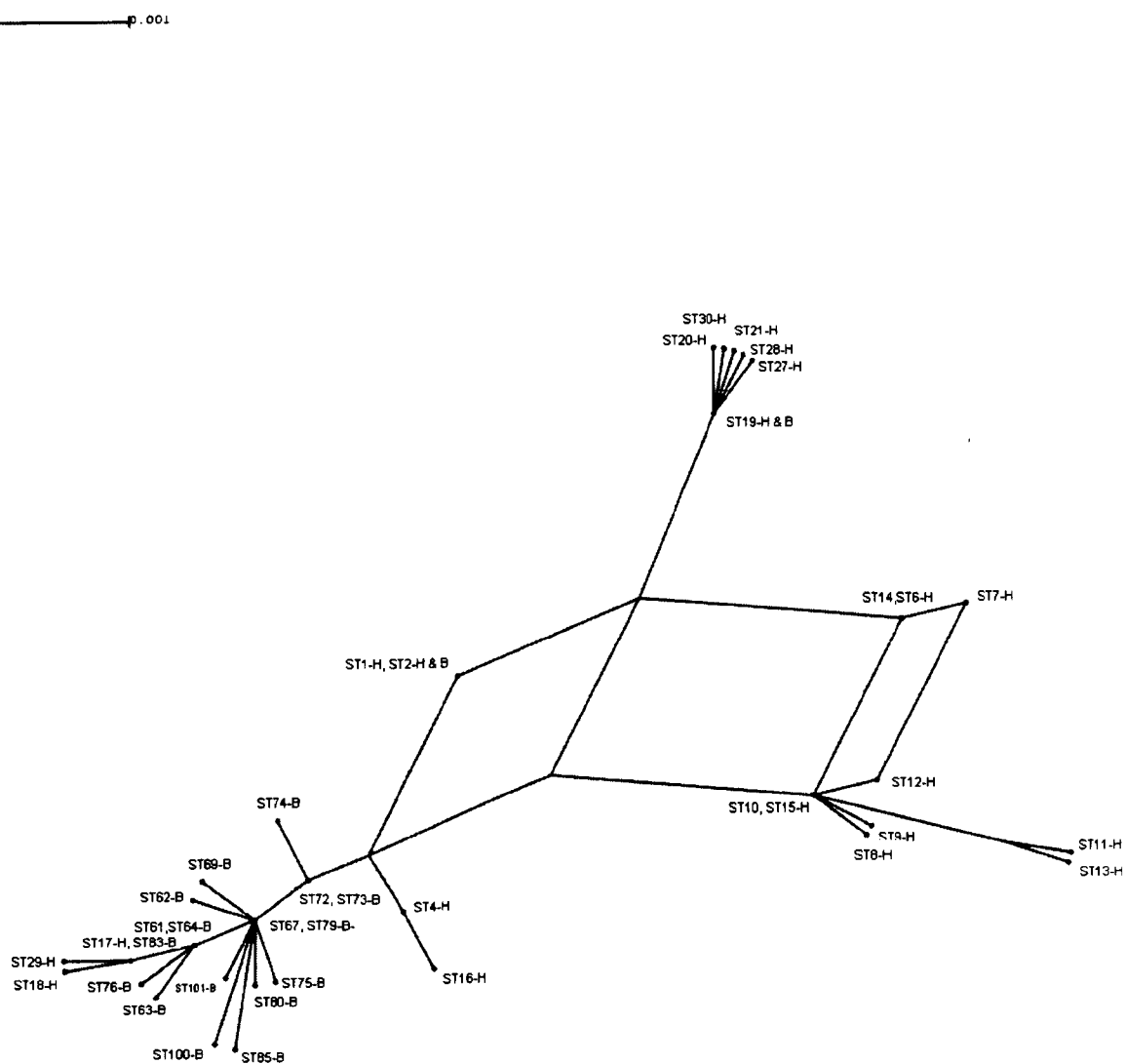


Figure 9.4. Tree drawn by split decomposition showing relationships between bovine and human GBS collections after removal of long branches.



9.5 DISCUSSION:

We have shown that populations of GBS from bovines and humans are largely discrete, which is consistent with previous work^{155,156}, but have clear areas of overlap. The phylogenetic evidence suggests that human ST-17, the hyperinvasive neonatal clone¹⁵⁷, has arisen from a bovine lineage.

In order to achieve this analysis, large carefully assembled collections of GBS isolates have been studied. The bovine isolates were collected from UK cases of bovine mastitis between 1950 to present day. The human isolates belong to a well-characterized global collection, which has been described in detail in Chapter 6.

Excluding the ST-23 complex, which is more distantly related, the remainder of the human GBS STs fall into one large group of related clusters. There is marked diversity in this group of clusters (Figure 9.4), demonstrated by the parallel branches of the split graphs, which reveal that the phylogenetic relationships are complicated and are indicative of recombination.

The most prevalent human STs, other than ST-17 are ST-1, ST-19 and ST-23. These STs have diverse capsular serotypes, exhibit more complicated phylogenetic relationships and include both carried and invasive isolates suggestive of opportunistic pathogenicity. Occasional bovine isolates are found within these STs.

In contrast, the bovine mastitis collection of isolates are generally lacking in diversity. Almost two-thirds of bovine strains (73 (65.8%)) are ST-67. ST-67 can be found within a cluster of strains, which includes the human ST-17 complex.

Previous molecular studies have suggested that isolates of bovine origin are of high diversity^{99,158}, which is inconsistent with the data presented here. This may reflect different typing approaches used or perhaps geographical bias, as all bovine isolates were from the UK. Furthermore, the bovine GBS isolates studied were collected from the 1950s onwards when pasteurisation and improved methods of hygiene in dairy farms were routine. For these reasons a more clonal population structure for bovine GBS may be expected.

The greater genetic diversity of the human lineages indicates that this may represent or be descended from a parent population of GBS. The clonal expansion evident for the bovine STs suggests more recent evolution from the parent population. The finding of the human neonatal hyperinvasive ST-17 complex within this cluster is a significant finding and is consistent with the concept that this lineage was acquired from the bovine subpopulation of GBS relatively recently.

In conclusion, it has been shown from this study that populations of GBS from bovines and humans are largely discrete, but have clear areas of overlap. The phylogenetic evidence indicates that human ST-17, the hyperinvasive neonatal clone, has arisen from a bovine lineage. The epidemiology of human neonatal GBS infection is that of recent and sustained emergence since the 1970s for

reasons that have never been fully elucidated. It is intriguing to postulate that the increase in GBS infection in human neonates may be at least in part due to the relatively recent introduction of the GBS genetic lineage corresponding to ST-17 complex into humans from cattle. The finding that ST-17 complex accounts for a proportion of adult carried isolates (Table 9.1) suggests it is now autonomously circulating within the human population. Further changes in animal husbandry are therefore unlikely to alter disease prevalence in neonates. Nevertheless this represents an example of a pathogen that has jumped the species barrier. Further investigation of this model will require more extensive sampling of bovine and human isolates and their characterisation by MLST and related techniques.

Chapter 10: Conclusions.

The aims of this project were to develop a multilocus sequence typing system for GBS. The system has been validated on a global collection of human GBS isolates isolated from carriage and invasive disease. The population biology of GBS was investigated on assembled collections of GBS representing human carriage and disease in neonatal and adult groups from a single geographical area (Oxford, UK). Finally, the relationship between human and bovine GBS has been explored.

The MLST system developed for GBS proved to be highly discriminatory when compared with the current gold standard typing method for GBS, capsular serotyping. A database of isolates and a website for GBS (www.sagalactiae.net) has been established and is in the public domain.

GBS is the most common cause of neonatal infectious disease in Oxford, with rates of infection of 0.94/1000 live births, which approximate to the published national statistics. GBS is a common pathogen in adults in Oxford, particularly in pregnant women (18.0/100,000) and the over 60 years age group (2.9/100,000). A large carriage study involving 748 pregnant women has been undertaken and has shown that 21.3% of women in the Oxford area are colonised vagino-rectally by GBS at the time of delivery.

A collection of almost 300 GBS isolates from asymptomatic pregnant women and neonatal invasive disease from a defined geographical area of the UK over a

similar time period was assembled. Analysis of population structure has shown that there are 6 major clusters of GBS, all of which contain isolates from carriage and neonatal invasive disease. The relationships between the major complexes was best depicted as a network rather than branching lines and was consistent with the presence of recombinational events occurring in the population of GBS that interfere with a tree like branching structure of the population.

One clonal cluster, ST-17 complex, was significantly associated with neonatal invasive disease (odds ratio 3.4, p-value 0.00003), particularly late onset infections (odds ratio 6.0, p-value 0.000002). Furthermore the invasiveness of this clonal complex appears to be independent of capsular type III. This raises the possibility that a factor other than capsular type III may be responsible for virulence of this clonal complex in neonates. The remaining GBS complexes were successful well-adapted complexes, as indicated by their prevalence in the population of both carriage and invasive isolates.

In contrast, hypervirulent clones were not identified in an investigation of GBS isolates from adults with invasive disease in the age group > 60 years. Clonal complexes that were prevalent in asymptomatic carriage were also successful as pathogens in this setting.

When a collection of bovine derived GBS was investigated and compared with a global collection of human isolates, the populations of GBS from bovines and humans were shown to be largely discrete but have clear areas of overlap. A

particularly interesting finding was that human ST-17 complex, the hyperinvasive neonatal clone, has arisen from a bovine lineage.

The epidemiology of human neonatal GBS infection is that of recent and sustained emergence since the 1970s for reasons that have never been fully elucidated. It is intriguing to postulate that the increase in GBS infection seen in human neonates may be at least in part due to the relatively recent introduction of the GBS genetic lineage corresponding to ST-17 complex into humans from cattle.

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Appendix 2: Documentation for Ethical Committee.

1. Letter confirming ethical approval
2. Patient information sheet
3. Consent form
4. Letter for General Practitioners.

Oxford Radcliffe Hospitals

NHS Trust

CENTRAL OXFORD RESEARCH ETHICS COMMITTEE

Manor House
Headley Way, Headington
Oxford OX3 9DZ

Tel: 01865 222547
Fax: 01865 222699

dms/em/C00.035

20 April 2000

Dr Nicola Jones
Department of Microbiology
John Radcliffe Hospital
Oxford OX3 9DU

Dear Dr Jones

Re: C00.035 – Carriage of Group B streptococci in pregnant women in the Oxford Radcliffe Trust

Thank you for your letter dated 10th April 2000, addressing the concerns raised by the Committee at their meeting on 3rd March. In accordance with the authority set out in the Terms of Reference, I am happy to confirm ethical approval and wish you every success with the study.

Please note:

- Ethical approval is valid for three years from the date of this letter. Annual updates of the progress of the research and a report of the outcome are required. (A reminder letter will be sent when these reports are due).
- No significant changes to the research protocol should be made without appropriate research ethics committee/chairman's approval. Any deviations from or changes to the protocol which increase the risk to subjects, or affect the conduct of the research, or are made to eliminate hazards to the research subjects, should be made known to COREC.
- COREC should be made aware of any serious adverse events.

- Whilst the study has received approval on ethical grounds, it is necessary for you to obtain management approval from the relevant Clinical Directors and/or Chief Executive of the Trusts (or Health Boards/DHAs) in which the work will be done.

I should be very grateful if you could send me a copy of any publication which may arise from this study.

Cont...

NB: Any research which will be conducted on NHS patients or staff, and which has been approved by a research ethics committee must carry the appropriate indemnity. May I remind you that COREC final approval is contingent on the appropriate indemnity being in place.

Yours sincerely,

Dr C J Chapman
Chairman
Central Oxford Research Ethics Committee

Central Oxford Research Ethics Committee (COREC)		
COREC No: C00.035		
Title of Project: Carriage of Group B streptococci in pregnant women in the Oxford Radcliffe Trust		
The following documents have been approved by COREC		
	Date/Version	Approved
Patient Information Sheet and Consent Form	20.4.00	✓
	20.4.00	✓
GP Letter	20.4.00	✓

Terms of Reference, Standard Operating Procedures and a list of members of the Ethics Committee are available from the Research & Development office on request.

INDEMNITY

The purpose of an indemnity arrangement for a researcher is to provide legal protection in the event of a researcher led unforeseen adverse circumstance, however minimal the risk, arising during the course of a research project. The indemnity applies to the Senior Investigator in the project and automatically covers any other generally more junior colleagues associated with the project. There are various types of indemnity dependent on the circumstances of the researcher and the nature of the research project. Staff employed in the NHS Trust Hospitals should ensure that they are properly protected by the appropriate indemnity approved by the Trust Chief Executive or Medical Director.



**Department of Microbiology
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20th April 2000
Version 1
COREC: C00.035

***PREVENTING INFECTIONS IN THE NEWBORN BABY
THE HORTON HOSPITAL GBS CARRIAGE STUDY
Information sheet for potential participants***

You are being invited to take part in a research study. Before you decide, it is important for you to understand why research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP, if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

Consumers for Ethics in Research (CERES) publish a leaflet entitled "Medical Research and You". This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 0BW.

Thank you for reading this.

What is the purpose of the study?

The Obstetric and Microbiology departments in the Oxford area are conducting a study over a twelve month period which aims to see how frequent a particular germ called GROUP B STREPTOCOCCUS is in pregnant women at the time of delivery of their baby.

Group B streptococcus, or GBS, is a germ (bacterium) which lives harmlessly in the vagina of healthy women. On rare occasions, GBS can spread to the newborn baby and cause severe infection.

Currently we do not know how many women in the UK have GBS. We estimate that it is roughly 15-20% of all women. It is currently not clear how doctors and midwives should be treating pregnant women in whom GBS is detected.

Why have I been chosen?

All women who are in the final few weeks of pregnancy will be asked to participate. Over the course of the year it is hoped that 1000 women will take part.

Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign a consent form. You are still free to withdraw at any time from the study without giving a reason. This will not affect the standard of care you receive.

What will happen if I take part?

If you agree to take part in the study, a single swab will be taken from the rectal and vaginal areas at the time of your first examination during your labour. This takes less than one minute and does not require an internal examination. It is a safe and easy procedure which would not harm you or your baby in any way. You will also be asked to fill in a brief questionnaire.

Will my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. Any information about you which leaves the hospital will have your name and address removed so that you can no longer be identified.

Your own GP will be notified of your participation in the study.

What will happen to the results of the research study?

The results of the study will be published in a medical journal approximately 12 months after the finish of the study. A copy of the results will be available from Dr Nicola Jones (address above). You will not be identified in any report or publication.

Who is organising and funding the research?

The Medical Research Council and Action Research, which are charitable organisations, are funding the research.

Who has reviewed the study?

The Central Oxford Research Ethics Committee has approved this study.

Contact for further Information

Further information can be given by Dr Nicola Jones and Dr Derrick Crook, Department of Microbiology, John Radcliffe Hospital, tel 01865 220125 or Mrs Yvonne Jones, Senior Midwife, Horton Hospital, tel 01295 229462.

Thank you for taking part in the study.

You will be given the information sheet and a copy of the consent form to keep.

**Department of Microbiology
John Radcliffe Hospital
Oxford OX3 9DU**

Tel 01865 220125
Fax 10865 220890

Corec Number: C00.035
20th April 2000

CONSENT FORM

***PREVENTING INFECTIONS IN THE NEWBORN BABY
THE HORTON HOSPITAL GBS CARRIAGE STUDY***

Researcher: Dr Nicola Jones, Microbiology

Please initial box

1. I confirm that I have read and understand the information sheet dated 20th April 2000 (version 1) for the above study and have had the opportunity to ask questions.

☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.

☐

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from the John Radcliffe Hospital where it is relevant to my taking part in research. I give permission for these individuals to have access to my records.

☐

4. I agree to take part in the above study.

☐

Name of Patient

Date

Signature

Name of Person taking consent
(if different from researcher)

Date

Signature

Researcher

Date

Signature

Please complete all 3 consent forms enclosed:- 1 copy for patient; 1 to be kept with hospital notes; 1 for researcher (send to Dr N Jones, GBS Carriage study, level 7 Dept of Microbiology JR Hospital).

Version 1
COREC: C00.035

20th April 2000

***PREVENTING INFECTIONS IN THE NEWBORN BABY
THE HORTON HOSPITAL GBS CARRIAGE STUDY
Information sheet for General Practitioners and Health Visitors***

Dear Colleague,

Your patient Date of Birth.....

Address.....

has kindly agreed to take part in our study.

Background to the study: Group B streptococcus is the most common infectious cause of neonatal morbidity and mortality in the UK. Currently we do not know how common GBS carriage is in pregnant women. It is hoped that the information gained from this study will be used in the formation of guidelines for the prevention of GBS disease in neonates.

Aims: To determine the carriage rate of GBS in women attending the antenatal clinic at the Horton Hospital, Banbury.

Research Protocol: Midwives are being asked to recruit women in the final trimester of pregnancy. An information sheet is available for potential participants. The midwife will explain the purpose of the study and ask the patient to complete a consent form and fill in a brief questionnaire. At the first routine examination in labour, the midwife will take a vagino-rectal swab and collect some patient details for a second questionnaire. The swab, consent form and questionnaires are sent to the JR hospital for analysis by hospital transport services.

The study has been approved by the Ethics Committee in Oxford (COREC C0.035). Results will be made available at the end of the study. For any queries please contact Dr N Jones or Dr D Crook, Department of Microbiology, John Radcliffe Hospital, Oxford, tel 01865 220125 or Mrs Yvonne Jones, Senior Midwife, Horton Hospital, Banbury, 01295 229462.

Yours faithfully,

Nicola Jones
Specialist Registrar Department of Microbiology

**APPENDIX
NOT COPIED**

**ON INSTRUCTION
FROM
THE UNIVERSITY**